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Reversing Anoikis Resistance in Triple-Negative Breast Cancer

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

During the second year of this idea expansion grant, we addressed **Aim1**, which was to determine if restoration of miR-200c and inhibition of miR-222 can enhance TNBC differentiation in 3D culture. Additionally, restoration of miR-200c decreases the amount of xCT protein, which regulates intracellular glutathione levels). We also completed tasks in **Aim 2** to **i**dentify the mechanisms by which TNBC cells resist anoikis. We confirmed that TNBC regulators of reactive oxygen species such as xCT and stress and inflammatory pathway genes including COX2 in suspension. Lastly, we determined that components of the kynurenine pathway (KP) increases when TNBC are surviving in suspension culture and restoration of miR-200c to TNBC reduces the rate limiting enzyme in this pathway (TDO2), another enzyme and also reduces the receptor for kynurenine (AhR). Lastly we have now identified splicing factors affected by restoration of miR-200c to TNBC and identify several critical splicing factors and splicing events altered by this miRNA that likely affect epithelial versus mesenchymal pheonotype (differentiation state). Our study continues to generate mechanistic and pre-clinical data necessary to determine if manipulation of these key miRNAs *or their downstream targets* has potential as a form of "differentiation therapy" for TNBC, for which there is currently no effective targeted treatment.

15. SUBJECT TERMS

Breast cancer, androgen receptor, estrogen receptor, growth factors, enzalutamide, endocrine resistance, targeted therapy

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1. INTRODUCTION:

For this idea expansion grant, we hypothesized that loss of miR-200c which represses many mesenchymal and neuronal genes and overexpression of miR-222, which targets estrogen receptor alpha are largely responsible for the highly dedifferentiated phenotype and aggressive behavior of claudin low TNBC and metaplastic breast cancers, and that manipulation of these miRNA or key targets could render such tumors less aggressive and more treatable. Normal epithelial cells or even well-differentiated breast carcinoma cells undergo detachment-induced apoptosis or "anoikis" and die when they are cultured in forced suspension , but triple negative breast cancer (TNBC) cells (except for the more epithelial-like Luminal androgen receptor (LAR)) subtype are dedifferentiated and they survive well in suspension and resist anoikis. In this **second year we completed tasks in Aim 1, and Aim 2 to identify the mechanisms by which miR-200c and miR-222 affect differentiation state and anoikis resistance, or survival in suspension.** We find that Dicer protein is significantly lower in TNBC clinical specimens than ER+ breast cancer and have submitted a paper on that topic (see appendix). **We also recently completed Task 4, which was to identify splicing factors altered by restoration of miR-200c in TNBC**. We requested and received a no cost extension because we were continuing to follow up on the splicing factors regulated by restoration of miR-200c and which genes they are acting upon. In this report we show some of that data since it has taken awhile to analyze the genome wide splicing events. **For Task 6** we confirmed at the protein level, that TNBC upregulate regulators of reactive oxygen species such as xCT and stress and inflammatory pathway genes including COX2 and downstream effector levels of prostaglandin E(2) (PGE(2)), also increase in suspension. T**o address Task 7**, we followed up on our observation that components of the kynurenine pathway (KP), such as the rate limiting enzyme TDO2 (that leads to production of the metabolite kynurenine and to production of the essential pyridine nucleotide, nicotinamide adenine dinucleotide (NAD(+)) increase when cells were surviving in suspension culture. The receptor that kynurenine binds to, the aryl hydrocarbon receptor, also increased in suspension. We postulated that kynurenine secreted by TNBC cells then binding to AhR in an autocrine manner, contributed to the ability of TNBC to resist anoikis and be more metastatic *in vivo*. For **Aim 3 Tasks 8 and 9 we performed an experiment to determine if treatment with an inhibitor of the enzyme TDO2, which is the rate limiting enzyme in the pathway that makes kynurenine, would render the cells less metastatic *in vivo*.** We published a paper on our findings regarding the effects of inhibiting this pathway on TNBC survival in suspension and metastasis (see appendix D'Amato and Rogers *Cancer Research* 2015).

By studying how miR-200c suppresses translation of these transcripts inappropriately expressed by carcinomas to facilitate anoikis resistance AND alters splicing events, we continue to generate mechanistic and pre-clinical data necessary to determine if manipulation of key miRNAs has potential as a form of "differentiation therapy" for TNBC. The work in this study may have great impact on the field of breast cancer therapeutics eventually because we have identified proteins that TNBC make only when they are in suspension that enable survival while in transit to metastatic sites. Identification of these genes/proteins was not possible via mining of data bases such as TCGA, which were generated with RNA from primary tumors, not tumor cells in circulation. Identification of genes and their protein products essential for "anchorage independence" or survival in suspension during metastatic spread will likely provide unique targets for TNBC therapy.

2. KEYWORDS: Breast cancer, anoikis resistance, xCT, COX2, TDO2, survival in suspension, metastasis, triple negative breast cancer (TNBC)

BODY:

3. ACCOMPLISHMENTS: Below each task in the official statement of work not addressed or completed in the first annual report is listed along with our the specific objectives, major activities, significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative) and other achievements. We include discussion of stated goals not met or tasks not fully completed. We include pertinent data and graphs in sufficient detail to explain significant results achieved. We have three manuscripts drafts in preparation almost ready to submit that relate to this project.

The objective of Aim 1 of this proposal is to determine if restoration of miR-200c and inhibition of miR-222 in TNBC can enhance differentiation in 3D culture and render TNBC more like the less aggressive luminal subtype. **What was accomplished under these goals?**

Task 2. Months 4-5. (75% completed) Determine if the protein products of targets that we know to be relevant in TNBC and change when we re-introduce miR-200c alone or combine with inhibition of miR-222 activity in 3D culture

We previously published that both restoration of miR-200c and antagonizing miR-222 increase Dicer levels. In order to do more work regarding Dicer in breast cancer we had to optimize an antibody for immunohistochemistry (IHC) because we had noticed some discrepancies in the literature regarding breast cancer, with different groups coming to different conclusions regarding the levels of Dicer in TNBC versus ER+ breast cancer. We have now worked out which antibody is optimal for Dicer protein detection.We previously published that miR-200c is reduced TNBC and miR-222 elevated in TNBC (REF). We now report that Dicer protein as detected by IHC is dramatically reduced in TNBC clinical specimens as compared to adjacent normal epithelium, while this is not the case in ER+ disease (see manuscript (Spoelstra NS et al. submitted to Modern Pathology in appendix).

Also as part of Aim 1 Task 2 we stated that we would determine, at the protein level, genes that increased in suspension and were possible targets of miR-200c. We find that TNBC upregulate regulators of reactive oxygen species such as xCT and stress and inflammatory pathway genes including *COX2* and downstream effector levels of prostaglandin E(2) (*PGE(2*)), which also increased in suspension. Lastly, we have also followed up on our observation that components of the kynurenine pathway (KP), such as the rate limiting enzyme TDO2 (that leads to production of the metabolite kynurenine and the essential pyridine nucleotide, nicotinamide adenine dinucleotide (NAD(+))) increase in suspension. Kynurenine, and the receptor that it binds to, the aryl hydrocarbon receptor, also increase in suspension. These factors contribute to the ability of TNBC to resist anoikis and be more metastatic in vivo. We demonstrated that knockdown of COX2 increases death in suspension as does knockdown or inhibition of TDO2 or AhR (in first annual progress report).

For **Aim 2 experiment 2 Task 7** we stated that we would determine if repression of the kynurenine pathway (KP) reverses anoikis resistance in TNBC cells. The KP pathway is the principal route of l-tryptophan catabolism leading to the formation of the essential pyridine nucleotide, nicotinamide adenine dinucleotide (NAD(+). This pathway has been implicated in immune suppression and tumor progression (6). Interestingly, we observed that 3 genes in this pathway (tryptophan 2,3-dioxygenase (TDO2), kynureninase L-kynurenine hydrolase (KYNU) and the aryl- hydrocarbon receptor (AHR) are among the 10 genes most highly upregulated by TNBC cells under conditions of forced suspension for 24 hrs. Our studies during this reporting period determined that TDO2 and KYNU proteins in TNBC increased in suspension culture (see manuscript in appendix (D'Amato and Rogers *et al* Cancer Research) and inhibition of TDO2 decreased secretion of kynurenine, activation of AhR, survival in suspension, and consequent lung metastasis. We published a paper on our findings (see appendix D'Amato and Rogers *Cancer Research* 2015). We noticed that the genes encoding these proteins contained predicted miR-200c target sites, so we tested now show that restoration of miR-200c reduces the levels of these transcripts **(Figure 1). T**his likely occurs via a predicted 3 binding sites (**Figure 2**), however, this remains to be tested.

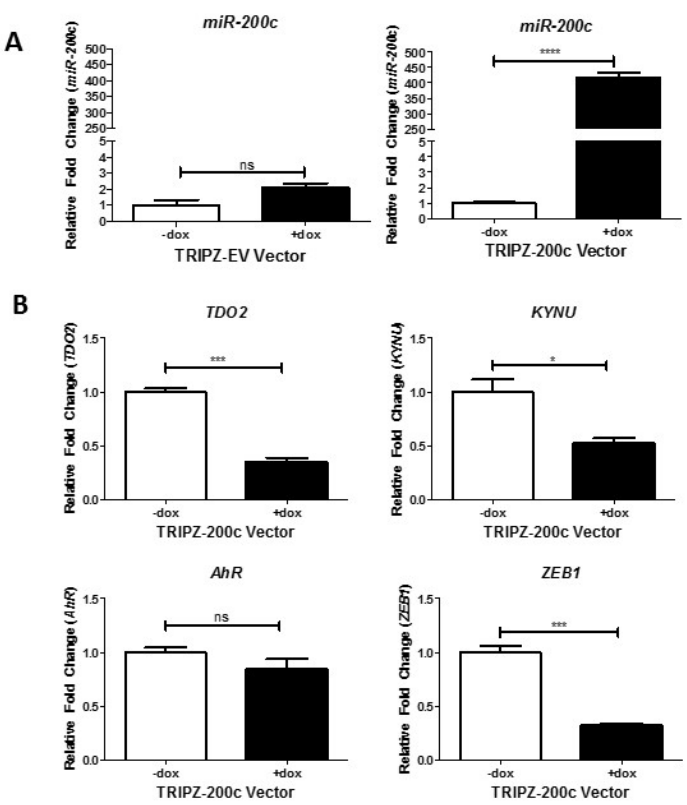
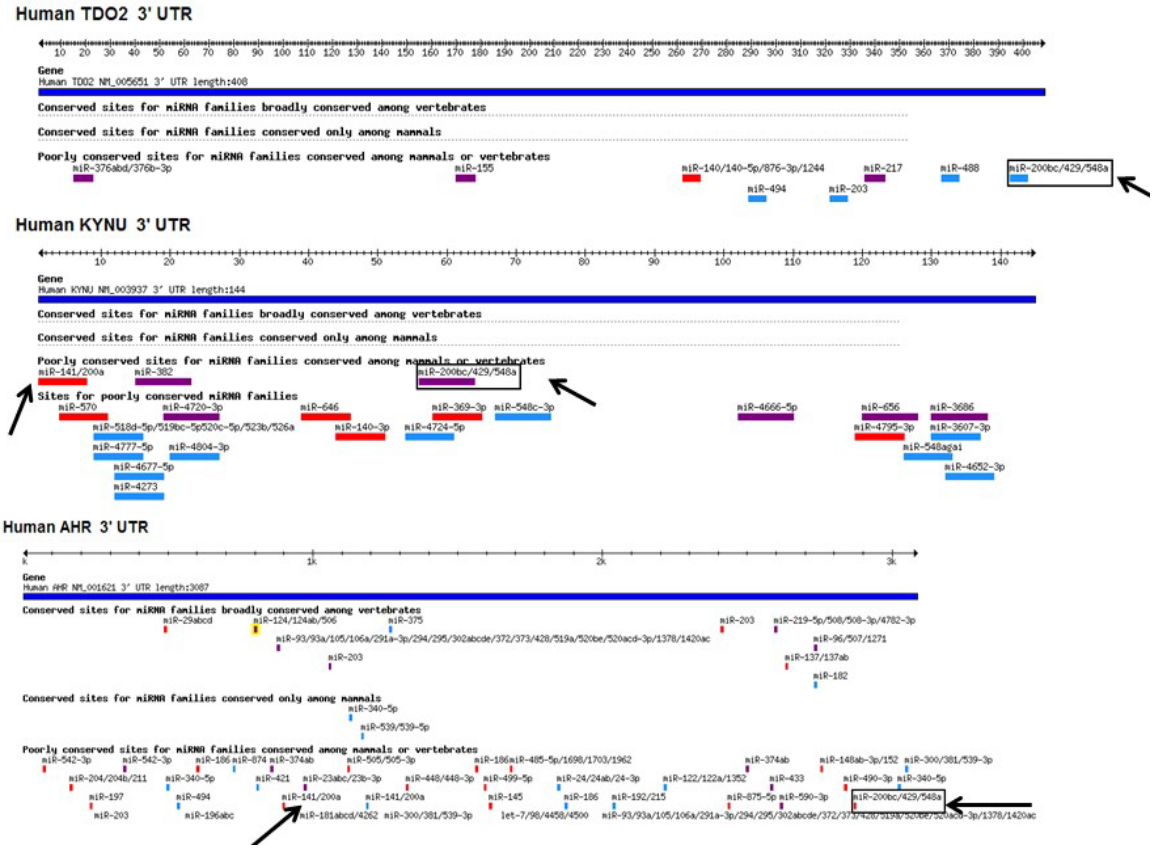


Figure 1. Doxycyclin inducible restoration of miR-200c decreases mRNA expression of Kynurenine Pathway Enzymes TDO2 and KYNU as well as AhR, the receptor for kynurenine the TNBC cell line BT549. (A) *Relative miR-200c levels following induction with doxycycline of a empty TripZ vector (EV) control or miR-200c inducible TripZ vector by doxycycline treatment for 48 hours.* ***(B)*** *Relative mRNA expression levels of Kynurenine pathway enzymes TDO2 and KYNU, the aryl hydrocarbon receptor (AhR), and the mesenchymal transcription factor ZEB1, a confirmed miR-200c target gene, following 48 hours of miR-200c induction by doxycycline.*

Figure 2. The TDO2, KYNU and AHR genes, which all increase when TNBC cells are surviving in forced suspension culture, all contain predicted binding sites for miR-200 family members in their respective 3'untranslated regions. *The program Targetscan was utilized to search for predicted target sites for TDO2, KYNU and AHR. The sites are designated with arrows. We can now clone these particular sites and mutate critical residues to determine if miR-200c or family members can no longer bind and affect these gene sites by cloning the wild type and mutant sequences downstream of luciferase in a reporter used for this purpose to definitively prove whether these genes are direct targets of miR-200c*



Task 3. Months 5-8. To test whether ER will be restored by antagonizing miR-222 (not yet initiated). We will stain for ER following antagonizing miR-222, since we and others published that ER is directly targeted by miR-222 (1, 7). Determine if tamoxifen or ICI reduces the number and size of colonies in colony formation assays. We have not started this aim yet because we have been following up more on task 2-4), but we will address this next year. We will stain for ER and if ERalpha protein is restored, we will treat cells with or without 10 nM E2 and measure ER regulated proteins progesterone receptors (PR) and PS2.

Task 4. Months 8-10. Identify splicing factors affected by miR-200c (90% completed). Figure 7 in the first annual report we showed RT-PCR results for two epithelial splicing factors that increase in suspension when miR-200c is induced in BT549 TNBC cells. Since then we have performed the proposed global analysis with inducible miR-200c. In order to determine which splicing factors were altered by miR-200c restoration, and which genes are differentially spliced, we utilized Affymetrix GeneChip Human Exon 1.0 ST arrays. **Figure 3** shows the quality control on the induced cells (measuring the successful induction of miR-200c in the inducible cells, but not in the empty vector control). Figure X also shows the observed increase in epithelial splicing factors and decrease in mesenchymal splicing factors as well as qRT-PCR confirmation on independent RNA.

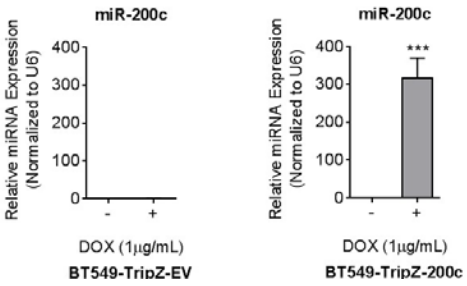
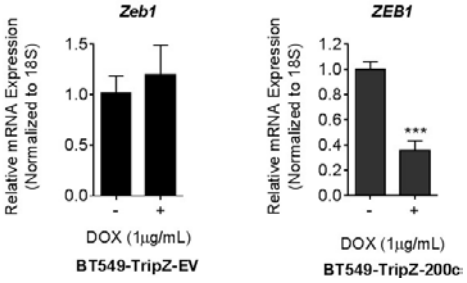
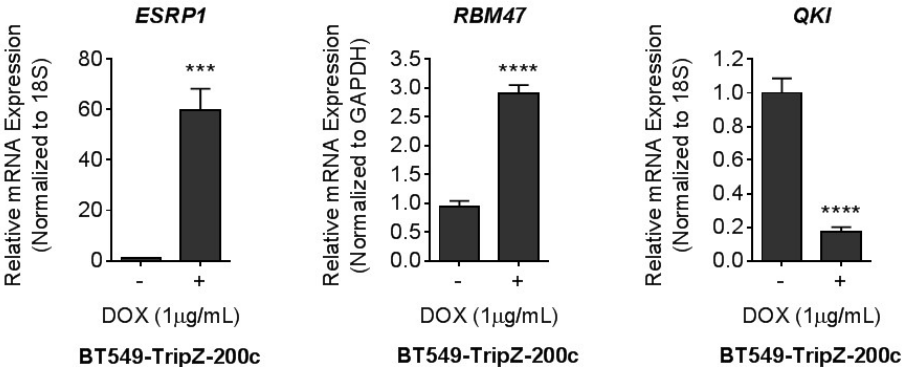


Figure 3. Inducible restoration of miR-200c to BT549 TNBC cells significantly alters RNA splicing factors. *BT549 cells stably transduced with the TripZ plasmid expressing a doxycycline inducible promoter upstream of miR-200c ore cells with stably integrated empty vector were treated with or without 1 μg/mL doxycycline for 48 hours and RNA was extracted. miR-200c levels were measured by TaqMan quantitative RT-PCR. Data was normalized to U6 and untreated (- DOX) expression levels; mean ± standard deviation, **** p < 0.0001 (top right). From the same RNA, qRT-PCR was performed for ZEB1, a known miR-200c target (bottom right). RNA in biological triplicate from induced and non-induced cells was sent to the University of Denver Genomics and Microarray Core where an Affymetrix Human Transcriptome 2.0 array was utilized to probe for changes in gene expression and splicing events following miR-200c induction. One-way ANOVA was performed at the gene level and at the probe level in order to identify differentially expressed genes as well as alternative splicing events.*



Splicing Factor		Fold Change	*p*-value
ESRP1	Epithelial splicing regulatory protein 1	**4.40**	4.00E-06
RBM47	RNA binding motif protein 47	**3.30**	0.0002
RBM23	RNA binding motif protein 23	1.34	0.0078
RBM38	RNA binding motif protein 38	1.19	0.0383
DAZAP2	DAZ associated protein 2	1.11	0.0240
QKI *	Quaking, KH domain containing RNA binding	**-4.20**	1.08E-08
RBFOX2*	RNA binding protein Fox-1 homolog 2	-1.23	0.0005
NOVA1*	Neuro-oncological ventral antigen 1	-1.21	0.0114
TIAL1*	TIA1 cytotoxic granule-associated RNA binding protein-like 1	-1.13	0.0093
CELF1*	CUGBP, Elav-like family member 1	-1.12	0.0118
DAZAP1	DAZ associated protein 1	-1.10	0.0191

* *miR-200c predicted targets (microRNA.org, mirdb.org)*



We have perform exon-level ANOVA examining differentially spliced transcripts that might affect differentiation state or tumor progression and find multiple alternatively spliced genes (several representatives shown in **Figure 4**). KIF1B encodes a pro-apoptotic motor protein that induces apoptosis by shuttling proteins to the nucleus. The full-length transcript encodes a tumor suppressor motor protein whose loss of expression has been implicated in progression of several cancer types including neurboblastoma and hepatocellular carcinoma. Interestingly, the full length form that increases in the miR-200c induced cells contains a region necessary and sufficient for apoptosis, suggesting a possible mechanism of restoration of anoikis sensitivity when miR-200c is induced (a phenomenon that we had previously reported (Howe EN *et al. Breast Cancer Research* 2011 and Howe EN et al PLoS ONE 2012). The truncated KIF1B isoform lacks a region necessary and sufficient for apoptosis, is a. The C-terminal truncated variant has impaired function and therefore is not as efficient at inducing apoptosis. Supervillin is a protein involved in focal adhesions, and the shorter splice variant archvillin is normally only expressed in muscle cells. GOLGA4 encodes a golgi protein involved in vesicle transport. It shuttles GPI-anchored proteins to the plasma membrane where they participate in cell-cell junctions. This transcript is interesting because one exon is spliced out when miR-200c is induced and another more 3' exon is retained, so we are still investigating the function of these regions.

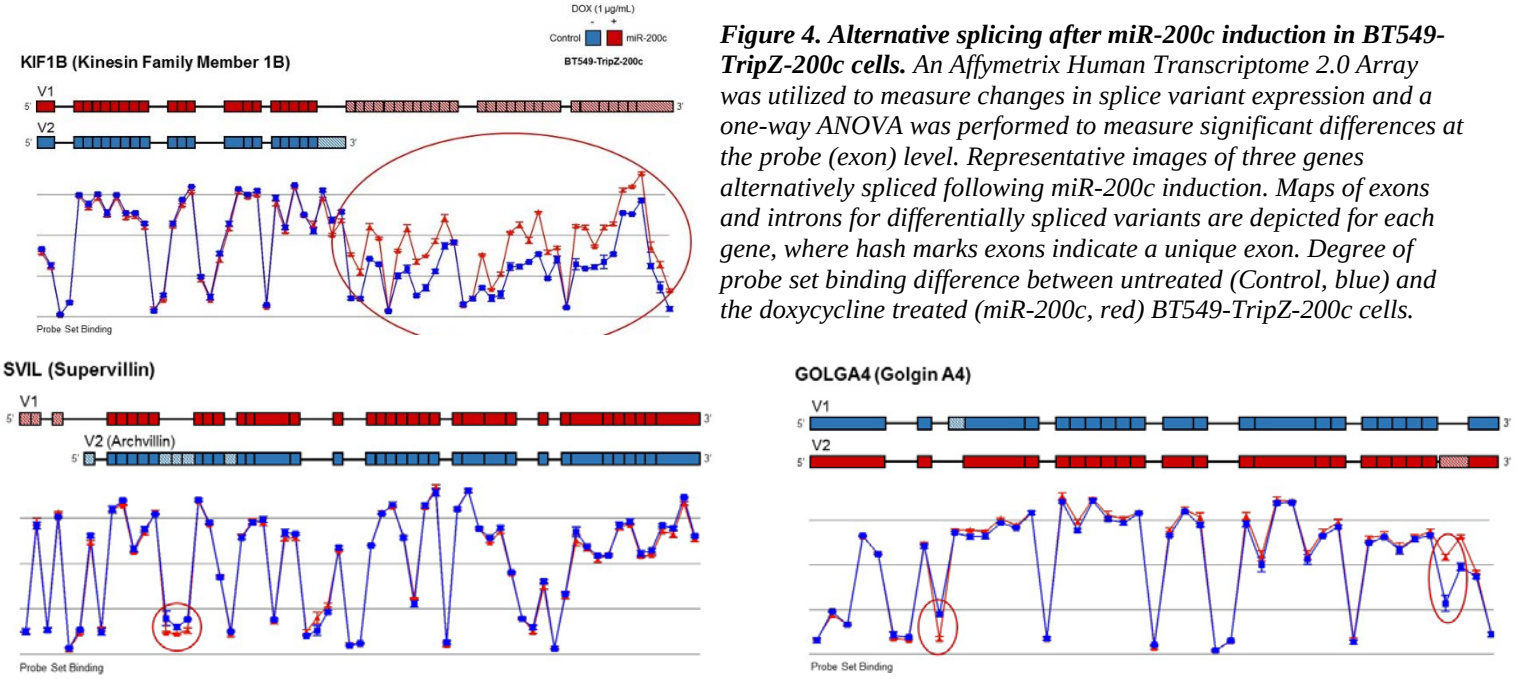
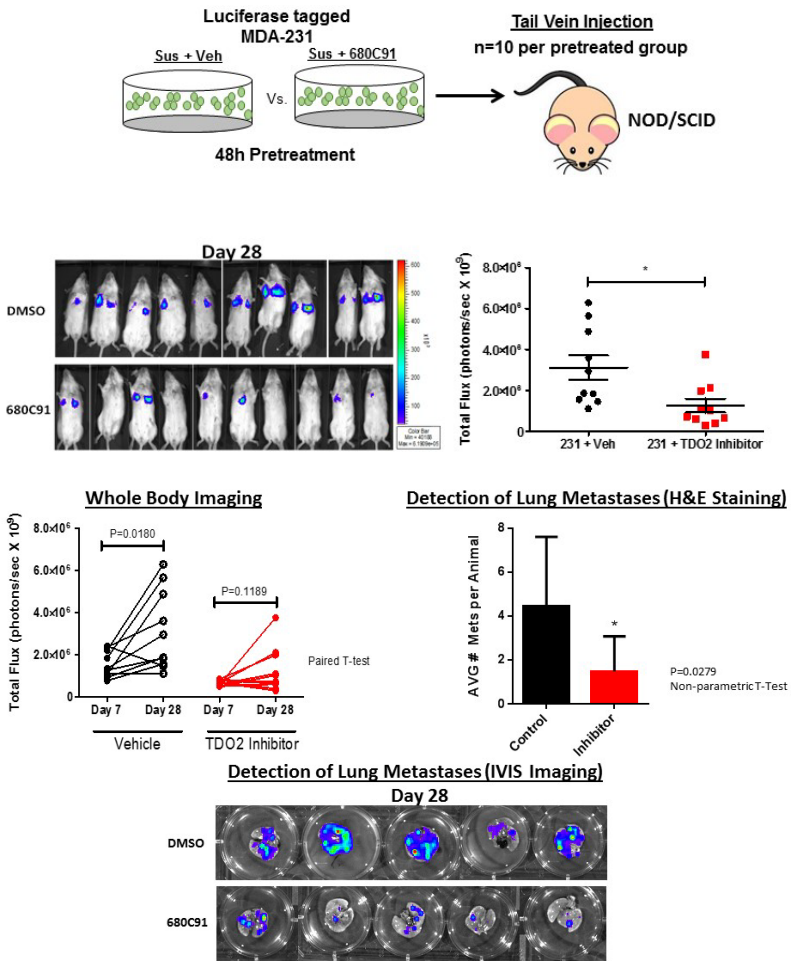


Figure 4. Alternative splicing after miR-200c induction in BT549-TripZ-200c cells. *An Affymetrix Human Transcriptome 2.0 Array was utilized to measure changes in splice variant expression and a one-way ANOVA was performed to measure significant differences at the probe (exon) level. Representative images of three genes alternatively spliced following miR-200c induction. Maps of exons and introns for differentially spliced variants are depicted for each gene, where hash marks exons indicate a unique exon. Degree of probe set binding difference between untreated (Control, blue) and the doxycycline treated (miR-200c, red) BT549-TripZ-200c cells.*

The objectives of Aim 3 tasks Task 8 and 9 were to perform *in vivo* experiment to determine if restoration of miR-200c or inhibition of one of its target genes would make TNBC less metastatic by reversing survival in suspension (anoikis resistance). Since intravenous delivery of miRNAs *in vivo* continues to be problematic for breast cancer because the miRNAs get caught up in the liver and kidneys and do not make it to the tumor cells, we had presented the possibility of testing an inhibitor of one of the targets of miR-200c in lieu of direct restoration of miR-200c, although restoration of miR-200c might ultimately be more powerful because it hits many targets. Because inhibitors of TDO have been tested in animals, we designed an experiment to test whether pretreatment with a TDO2 inhibitor would cause TNBC tumor cells to die in transit and not thrive as well at a metastatic site. Figure 5 shows results that we published in the Cancer Research paper attached in the appendix that do show that inhibiting this important miR-200c target does significantly decrease lung metastases.

Figure 5. Inhibition of TDO2 decreases lung metastasis. *Luciferase-tagged MDA-231 cells were pretreated with either vehicle control or TDO2 inhibitor for 48h in forced-suspension culture for 48h. Following, 250K viable cells were injected per mouse and 10 mice were used per pretreatment group. This tail-vein injection model tests late stage metastasis, involving survival in the vasculature, exiting the bloodstream, and growth at secondary site, in this case the expected secondary site is the lung. Mice were then imaged using IVIS imaging, which detects luciferase expressing cells in the animal. By the first day of imaging, Day 7, we already see an effect of pretreatment with the TDO2 inhibitor on lung colonization. This effect was we already see an effect of pretreatment with the TDO2 inhibitor on lung colonization. This effect was maintained through Day 28. When tumor outgrowth was measured by IVIS imaging, the cells pretreated with the TDO2 inhibitor did not significantly grow during the study. Significantly fewer metastases per animal by H&E staining of extracted lung tissue were detected. Luciferase signal as a measure of tumor cell viability in lungs when imaged ex vivo on Day 28 show fewer viable cells in the treated group.*



References:

1. Howe EN, Cochrane DR, Cittelly DM, Richer JK. miR-200c targets a NF-κB up-regulated TrkB/NTF3 autocrine signaling loop to enhance anoikis sensitivity in triple negative breast cancer. PLoS One. 2012;7(11):e49987. PubMed PMID: 23185507; PubMed Central PMCID: PMC3503774.
2. Howe EN, Cochrane DR, Richer JK. Targets of miR-200c mediate suppression of cell motility and anoikis resistance. Breast Cancer Res. 2011 Apr 18;13(2):R45. PubMed PMID: 21501518; PubMed Central PMCID: PMC3219208.
3. D'Amato NC, Rogers TJ, Gordon MA, Greene LI, Cochrane DR, Spoelstra NS, Nemkov TG, D'Alessandro A, Hansen KC, Richer JK. A TDO2-AhR Signaling Axis Facilitates Anoikis Resistance and Metastasis in Triple-Negative Breast Cancer. Cancer Res. 2015 Sep 11; PMID:26363006 PMCID: PMC4631670

4. IMPACT:

- **What was the impact on the development of the principal discipline(s) of the project?** This work may have great impact on the field of breast cancer and cancer research and oncology practice eventually, because we have identified genes and their protein products that TNBC make only when they are in suspension. These findings are of interest in cancer biology because we never would have known that the

most aggressive TNBC subtype of breast cancer could make these different genes and proteins that likely facilitate metastasis if we did not do our global screening of what they are making in suspension. The data are different from the data in the TCGA database because those are genes expressed in the primary tumor, not cells that can escape from the primary and survive in transit to a secondary metastatic site. If we identify what tumor cells are using to survive in suspension and if these things are being inappropriately allowed to be expressed because due to microRNA alterations, then can we can potentially use miRs to achieve "differentiation therapy" via altering a whole pattern of gene expression and possibly even alternative splicing events by inducing expression of epithelial spicing factor. Knowledge of these proteins that are facilitating survival in suspension, may therefore provide unique, previously unidentified targets for TNBC therapy.

What was the impact on other disciplines? Our findings and the idea that cancer cells may turn on totally different genes and proteins when they are in suspension is novel and may be pertinent to other cancers. It also brings up the point that miRNA can target different genes at different stages of tumor progression or different stages of development.

- **What was the impact on technology transfer?** None to date.

What was the impact on society beyond science and technology? I have given reports of our research to both scientific and lay audiences to improve knowledge regarding how and why triple negative breast cancer is so aggressive and how we are elucidating unique ways to potentially target this subtype of breast cancer that has a high mortality rate, particularly when it becomes resistant to chemotherapy.

- **What do you plan to do during the next reporting period to accomplish the goals?**

For the in vivo experiments, we are a little behind because we wanted to think about our best strategy. We did BT549 by tail vein injection, but we want to do the MDA-MB-231 that metastasize from the orthotopic site. However, we could not select a population that showed good induction of miR-200c in a non-leaky manner.. We will also finish verifying and confirming the effects of miR-200c on splicing factors and splicing events.

6. CHANGES / PROBLEMS:

a. **Changes in approach and reasons for change**: None

b. **Actual or anticipated problems or delays and actions or plans to resolve them**: There was a delay in analyzing the splicing factor and spicing event data so we asked for and received the no cost extension. As shown in this report, we have figured out how to appropriately analyze the splicing data. Now we just need to form some confirmatory experiments and we can write up these novel data in a manuscript. We are also still working on getting these miRNA into TNBC patient derived xenografts (PDX). We hope to do this during the time of our no-cost extension

c. **Changes that had a significant impact on expenditures**. The delays above led to our asking for the no cost extension.

d. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents. None to report

e. Significant changes in use or care of human subjects. None

f. **Significant changes in use or care of vertebrate animals.** None

g. **Significant changes in use of biohazards and/or select agents.** None

6. REPORTABLE OUTCOMES and PRODUCTS

What opportunities for training and professional development has the project provided?

Student Thomas Rogers wrote an NRSA on this topic and it received a likely fundable score, but we have not heard the final word on it.

Student Thomas gave a talk that won the best talk by a student/postdoc award at the International Society for Tryptophan Research meeting 2015 September 16–18, 2015, at Van Andel Research Institute in Grand Rapids, Michigan (USA)

Lectures by Dr. Richer on this topic include:

March 2014 "Anoikis Resistance in Breast and Ovarian Cancer Progression" University of Florence Dept. Experimental and Clinical Biomedical Sciences, Biochemistry, Human Health Medical School Guest of Professor Paola Chiarugi

2015 Nov 6 "When projects collide or wow, maybe we are on to something!" Cancer Biology Graduate Program Annual Retreat

a. How were the results disseminated to communities of interest?

Dr. Richer was invited to speak at an international meeting and laboratory in Florence, Italy March 26-28 2014. For the meeting I talked about what we have learned regarding anoikis resistance in both breast and ovarian cancers since TNBC and serous ovarian cancers have many similarities including some of the things we have found to be important for survival in suspension, which is likely why these two diseases metastasize so readily.

Richer, JK visiting lecturer "Anoikis Resistance in Breast and Ovarian Cancer Progression" University of Florence Dept. Experimental and Clinical Biomedical Sciences, Biochemistry, Human Health Medical School Guest of Professor Paola Chiarugi March 2014.

Dr. Richer also gave a talk for the University of Colorado Cancer Center on this research:

2014 March 21 Cancer Center Hormone Related Malignancies Retreat –"Mechanisms of Anoikis Resistance During Breast and Ovarian Cancer Progression"

Funding applied for based on work supported by this award:

I applied for and was awarded a Breakthrough Award Level 2 grant "Targeting tryptophan catabolism: a novel method to block triple negative breast cancer metastasis." I had noted that some of the genes mentioned in this grant as potential targets of miR-200c were all in the tryptophan catabolism pathway and a metabolite of that pathway, kyneurine, was recently found to be a ligand for the arylhydrocarbon receptor (AhR), which we also find to be likely targeted by miR-200c (see this report) and is increased in suspension. Interestingly, AhR is not only found in the tumor cells themselves, but is also found in immune cells. Kynurenine (KYN) can act as an immunosurpressive agent by binding to AhR in various immune cells. Thus the **Breakthrough Award goal** is to test the new hypothesis that TNBC can make this ligand (KYN) that can act in a paracrine fashion to effectively shut down the immune system to allow the TNBC to evade immune attack. With a team of collaborators, including a tumor immunologist and an oncologist that specializes in young women's breast cancer, we will test our theory. The initial confirmation that the components of the tryptophan catabolism pathway, such as the rate limiting enzyme TDO2, were up not only at the gene level, but also the protein level when TNBC are cultured in forced suspension and that it and AhR-had miR-200c predicted binding sites came from this Idea Expansion grant. However, at the time that we wrote the

idea expansion grant we were only thinking of autocrine effects of KYN, not its effects on the immune system that could potentially explain why TNBC is such an aggressive subtype that often recurs and is lethal within 5 years of diagnosis. The fact that this pathway is targetable makes this particularly exciting and if inhibiting this pathway can render the immune system more able to recognize and effectively attack TNBC tumor cells, then it is a good target for therapy on more than one front.

PRODUCTS:

Inventions, patent applications, and/or licenses. None

Manuscripts:

Published:

D'Amato NC, Rogers TJ, Gordon MA, Greene LI, Cochrane DR, Spoelstra NS, Nemkov TG, D'Alessandro A, Hansen KC, Richer JK. A TDO2-AhR Signaling Axis Facilitates Anoikis Resistance and Metastasis in Triple-Negative Breast Cancer. Cancer Res. 2015 Sep 11; PMID:26363006 PMCID: PMC4631670

Submitted:

Spoelstra NS et al. Dicer expression in estrogen receptor positive versus triple negative breast cancer submitted to Modern Pathology, Dec. 2015

- **Other Products**

We have the inducible miR-200 BT549cells now (TripZ lentiviral vector) with stably introduced miR-222 miR-ZIP.

We have a data bank of genes that change in TNBC breast cancer cells with restoration of miR-200c and a list of genes that are differentially spliced.

We have formalin fixed paraffin embedded TNBC lines in culture in attached versus suspension culture.

We have two TNBC patient derived xenograft tumors.

Research material: we have many primers and antibodies now to the genes/proteins of interest.

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS:

What individuals have worked on the project?

i. Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Personnel	Role	Percent Effort	Nearest Person Month Worked	Contribution to Project	Funding Support: DOD Contract W81XWH-13-0222
Jennifer Richer, PhD	Partner Principal Investigator	25%	3	Oversees all experiments –, writes reports and edits manuscripts	X
Kiel Butterfield	Technician	100%	2	Since her hire in the lab in June 2014, performs tissue culture, support for attached versus suspended experiments,	X
Thomas Rogers	Graduate Student	100%	2	Began work on this grant in August, 2014, has performed experiments to test for alterations in TNBC cells following miRNA manipulations and suspen-sion culture	X

ii. **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? No**

b. **What other organizations were involved as partners?** None

8. SPECIAL REPORTING REQUIREMENTS - none

9. APPENDICES:

Abstract presented by postdoctoral fellow Jessica Christenson for our Cancer Biology Graduate Program

Manuscript submitted to Modern Pathology on Dicer and miR-200c in breast cancer

Published paper in Cancer Research on role of TDO2 in breast cancer anoikis resistance and metastasis.

miR-200c regulates the expression of splicing factors to promote the epithelial phenotype in triple-negative breast cancer

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Background: Small non-coding microRNAs (miR) regulate gene expression post-transcriptionally and are known to play critical roles in a variety of cellular processes. miR-200c, in particular, is known for its role in regulating breast cancer progression. The epithelial to mesenchymal transition (EMT) is a critical step necessary for the initiation of the metastatic cascade. Specifically, EMT causes gene expression changes that result in major phenotypic alterations that allow cells to detach from the basement membrane and survive in transit to distant sites. miR-200c is considered a 'guardian' of the epithelial phenotype and negatively regulates multiple mesenchymal and neuronal genes that contribute to EMT. In addition to changes in overall gene expression, alterations in gene splicing have also been shown to contribute to EMT. Preliminary data from ovarian cancer cells suggest that miR-200c regulates alternative splicing through targeting of splicing factor expression. The purpose of this study was to investigate if and how miR-200c alters gene splicing patterns and splicing factor expression in triple-negative breast cancer (TNBC).
Methods: The TNBC line BT549 was transduced with the TripZ plasmid expressing a doxycycline inducible promoter upstream of miR-200c. To induce miR-200c, 1 μg/mL doxycycline was applied to the cells for 48 hours and RNA was extracted from treated and non-treated cells. RNA in biological triplicate from induced and non-induced cells was sent to the University of Denver Genomics and Microarray Core where an Affymetrix Human Transcriptome 2.0 array was utilized to probe for changes in gene and splice variant expression after miR-200c induction. One-way ANOVA was performed at the gene level and at the probe level in order to identify differentially expressed genes as well as alternative splicing events. Quantitative RT-PCR and Western analysis were used to verify changes in gene and protein expression following miR-200c induction.
Results: Microarray data confirmed that the induction of miR-200c in BT549 cells leads to an increase in epithelial markers, such as E-Cadherin, and a decrease in markers of the mesenchymal phenotype, such as Zeb1. In addition, expression of ESRP1 and RBM47, splicing factors thought to be characteristic of epithelial cells, were upregulated following miR-200c induction, whereas QKI, a predicted miR-200c target and splicing factor more commonly expressed in cells of mesenchymal origin, was downregulated following miR-200c induction. Microarray analysis also suggested that induction of miR-200c leads to alternative splicing of multiple genes, including Zeb2 and the splicing factor RBM47 itself.
Conclusions: The role miR-200c in maintaining the epithelial phenotype is well established, and miR-200c is known to regulate many genes involved in EMT. However, this study is the first to establish a direct link between miR-200c and alternative splicing. In addition to directly targeting genes, miR-200c may also influence cellular processes by altering expression of factors responsible for alternative splicing. Further research will determine the mechanisms by which miR-200c alters splicing factor expression and elucidate the particular splicing patterns associated with miR-200c and the more epithelial state, including their functional relevance to the aggressive clinical behavior of the TNBC subtype of breast cancer.

Dicer Expression in Estrogen Receptor Positive versus Triple-Negative Breast Cancer: An Antibody Comparison

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Running title: Dicer Antibody Comparison in Breast Cancer

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Abstract:

Dicer is an RNase III enzyme responsible for cleaving double stranded RNAs into siRNAs and miRNAs, which either target mRNA transcripts for degradation, or inhibit translation. Dicer expression has been examined as a prognostic indicator in various cancers including breast cancer, with contrasting results at both the mRNA and protein level.

Here we compare three different Dicer antibodies: Abcam 4A6, Abcam ab5818, and Sigma HPA000694, using immunohistochemistry and western blot analyses. All three antibodies were able to detect higher levels of endogenous Dicer in T47D versus BT549 cells, as well as high levels of exogenous Dicer compared to non-transfected cells by western blot. Dicer was detected predominantly by these antibodies in the cytoplasmic extract of cells with minimal staining in the nuclear fraction. All three antibodies detected higher Dicer expression in estrogen receptor positive breast cancers versus triple-negative breast cancer, but had very different staining patterns by immunohistochemistry (intensity levels, percent positivity, and nuclear/cytoplasmic localization) within the same tumor samples. These differences were even more pronounced in xenograft tissues.

Antibody ab5818 was chosen for its sensitivity and specificity to stain a cohort of n=25 estrogen receptor positive and n=21 triple negative breast cancers, and Dicer expression was significantly higher in estrogen receptor positive versus triple-negative breast cancer (average score of 266.2 and 88.9 respectively, $p < 0.0001$, unpaired t-test). Dicer expression was also significantly higher in adjacent normal breast versus triple-negative breast cancer ($p<0.0001$, paired t-test, n=18 pairs), but was not significantly different between estrogen receptor positive tumor and adjacent normal breast.

A difference in antibody performance may be a confounding factor for the contrasting results observed in the literature by immunohistochemistry. As Dicer becomes more clinically relevant, further antibody optimization and standardization will be required.

Introduction

Human DICER1 is an RNase III enzyme responsible for cleaving double stranded RNAs (dsRNAs) into small interfering RNAs (siRNA) or microRNAs (miRNA). These small RNAs are then incorporated into a multiprotein RNA-induced silencing complex (RISC) which uses them as templates for targeting specific mRNAs leading to their degradation or inhibiting their translation. siRNAs and miRNAs play major roles in both development and disease (1).

DICER1 is known to be a haploinsufficient tumor suppressor as dysfunctional miRNA processing leads to enhanced transformation and tumorigenesis (2-4). However, Dicer levels studied in relation to tumor progression and outcome in various cancers have demonstrated conflicting results (5-8). For example, in breast cancer, a gradual loss of Dicer protein with disease progression was observed by immunohistochemistry (IHC) in clinical breast cancer specimens (normal > ductal carcinoma in situ (DCIS) > invasive carcinoma > nodal metastases) (9). In contrast, other studies have reported higher Dicer expression in nodal metastases compared to primary tumors by qPCR (10), and IHC (11).

Overall, estrogen receptor alpha positive (ER+) breast cancers have a better prognosis than triple-negative breast cancers (TNBC), that by definition lack ER, progesterone receptor (PR), and amplification of human epidermal growth factor receptor 2 (HER2) (12-14). Our group has previously published that Dicer protein expression is higher in ER+ versus TNBC cell lines, and that increasing miR-200c to levels found in ER+ lines results in dramatically increased Dicer protein. Inhibition of miR-221/222 in TNBC also increases Dicer since these miRNA directly target Dicer mRNA at sites in its 3'UTR (15). In addition, we demonstrated that when miR-200c was restored to human TNBC lines to levels observed in ER+ lines, production of the mature forms of other miRNAs, that like the miR-200 family were higher in ER+ luminal lines, also increased (15).

To validate our findings in clinical specimens of ER+ and TNBC, we sought to evaluate the performance of Dicer antibodies for IHC on formalin-fixed paraffin-embedded (FFPE) specimens. Dicer monoclonal antibodies 13D6, 4A6 (made from the same immunogen as 13D6) (Abcam), and polyclonal antibodies ab5818 (Abcam) and HPA000694 (Sigma) were compared using western blot and IHC analyses. To study differences in the qualities of these antibodies, we examine both endogenous and overexpressed Dicer, and manipulate endogenous Dicer expression via restoration of miR-200c. We also test a cohort of ER+ breast cancer and TNBC for Dicer protein expression by IHC. While the antibodies used performed similarly for western blot analyses, major differences were observed in cellular staining patterns of Dicer by IHC, highlighting the necessity for rigorous antibody performance evaluation before definitive conclusions can be drawn regarding Dicer expression in ER+ versus negative disease, or during disease progression. Based on data obtained with the Dicer antibody of choice following performance evaluation, we conclude that Dicer protein is significantly lower in TNBC than in ER+ breast cancer or adjacent non-involved breast epithelium.

Materials and Methods

Human Tissues: ER+ Breast Cancer: Postmenopausal women (n=25) with newly diagnosed ER+ breast cancer, stage II/III, T2-3 were included in this study. Core needle biopsies were taken prior to endocrine therapy and subjects who responded to endocrine therapy were included for this analysis. The protocol (01-627) was approved by the Colorado Multiple Institutional Review Board (COMIRB) and informed consent was provided by all patients. Triple-Negative Breast Cancer: Patients (n=21) ranged in age from 19-72 years old with a mean age of 47.44 ± 12.03 years. All tumors were grade 3 and negative for ER, PR and HER2 (COMIRB protocol 04-0066).

Cell Culture: T47D breast cancer cells, which are ER+ and belong to the Luminal A subtype, were grown in MEM, 5% fetal bovine serum (FBS), non-essential amino acids (NEAA), insulin, and penicillin/streptomycin (p/s). The TNBC cell line BT549 was grown in RPMI, 10% FBS, and insulin. HEY ovarian cancer cells were grown in RPMI, 10% FBS, L-glutamine, and p/s. HEK293FT human embryonic kidney cells were grown in DMEM containing 10% FBS. All cells were maintained at 37°C and 5% CO_2. All cells were fingerprinted for authenticity using the Identifiler DNA profiling kit (ABI, Grand Island, NY, USA) at the University of Colorado Cancer Center Sequencing Core Facility.

miR-200c Inducible Cells: BT549 cells were transduced with inducible lentiviral vector pTRIPz-RFP encoding the precursor sequence for miR-200c (pTRIPz-200c). Stable expression was selected using puromycin, and a single clone of BT549-TripZ-200c, showing a robust inducible expression of miR-200c and little background, was used in all subsequent experiments. BT549-TripZ-200c cells were plated at a density of 8×10^5 cells per 10cm dish, and miR-200c expression was induced with 1 µg/mL doxycyline (DOX) for 48 hours.

miR-200c Inducible Xenograft Tumors: These methods are previously described in (16). Briefly, HEY ovarian cancer cells expressing DOX-inducible miR-200c were grown in NOD SCID mice, and miR-200c expression was activated with 2 g/L DOX in drinking water. After 21 days, mice were euthanized by CO_2 asphyxiation and tumors were excised. Portions of each tumor were either frozen or formalin-fixed and paraffin-embedded. The University of Colorado Institutional Animal Care and Use Committee (Aurora, CO) approved these experiments.

Antibodies: Primary antibodies to Dicer were optimized and used at the following concentrations: mouse monoclonal 13D6 (1:25 for IHC), 4A6 (1:25 for IHC, and 1:50 for western blot (WB)), rabbit polyclonal ab5818 (1:50 for IHC and WB) (Abcam, Cambridge, MA, USA), (note: ab5818 is currently sold as PA5-19437 ThermoFisher, Grand Island, NY, USA) and rabbit

polyclonal HPA000694 (1:50 for IHC, 1:500 for WB) (Sigma-Aldrich, St. Louis, MO, USA). Additional antibodies used for western blot include α-tubulin clone B-5-1-2 (Sigma), and TOPOI (C-21) (Santa Cruz Biotechnology Inc., Dallas, TX, USA).

Blocking Peptide: A blocking peptide was commercially available for antibody ab5818 (Abcam ab24556). Peptide at 10X the concentration of antibody was incubated with antibody ab5818 rotating overnight at 4°C prior to use on tissue or western blot.

Immunohistochemistry: Sections were cut at 4µm and heat immobilized for 1 hour at 60C on charged glass slides. Sections were deparaffinized in three changes of xylene and a series of graded ethanols. Heat induced epitope retrieval was performed in a Decloaking Chamber (Biocare Medical, Concord, CA, USA) at 125°C for 5 min. in 10mM citrate buffer pH 6.0. Sections were blocked in 3% H_2O_2 followed by 2.5% normal horse serum before incubation for 1 hour with primary antibody. Tris buffered saline with Tween (0.05%) was used for all washes, and the Vectastain Universal Elite ABC horseradish peroxidase (HRP) kit (Vector Laboratories, Burlingame, CA, USA) was used for antibody detection, followed by DAB+ (Dako, Carpinteria, CA, USA) for chromagen visualization.

IHC Score: All clinical breast cancer slides were analyzed by a pathologist (PJ) for intensity (0-4 scale) and % positive cells stained, and these were multiplied to obtain a score.

Dicer Overexpression: HEK293FT cells were plated at 7.5 X 10^5 cells per 10cm dish. The following day, cells were transfected with media plus transfection reagent without DNA (mock) or 2µg of pCMV-Entry plasmid containing the 5769 bp open reading frame of Human DICER1, ribonuclease type III, transcript variant 2 (TruORF® OriGene Technologies, Rockville, MD, USA) using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA). After 48h, cells were harvested for protein analysis.

Western Blot: The NE-PER Nuclear and Cytoplasmic Extraction kit (ThermoFisher) was used for nuclear/cytosolic protein extraction. Whole cell protein extracts were prepared using RIPA lysis buffer, and 50 or 90µg of protein was separated by SDS-PAGE and transferred to a PVDF membrane. 3% BSA in TBST (0.1% Tween) was used for all blocking and antibody dilution. Secondary antibodies used were goat anti- mouse or rabbit Alexa Fluor® 680 (Molecular Probes, Grand Island, NY, USA). All blots were scanned using an Odyssey infrared imager (LI-COR Biosciences, Lincoln, NE, USA).

qRT-PCR: Total RNA was collected using the RNeasy Plus kit (Qiagen, Valencia, CA, USA). Mature miR-200c (#002300) and U6 snRNA (#001973, used as a house-keeping gene for normalization) were reverse transcribed using the TaqMan microRNA Reverse Transcription Kit and expression levels were analyzed using the TaqMan Universal PCR Master Mix from Life Sciences (ThermoFisher).

Statistics: The Mann-Whitney non-parametric t-test was used to compare unpaired groups of data, and a paired t-test was used in cases where data were paired. Both analyses were two-tailed.

Bioinformatics: Dicer gene expression data was obtained from www.oncomine.org, using the Curtis et al dataset (METABRIC dataset) (17). A total of 1950 patients were included in this analysis, with 440 patients having ER- tumors and 1510 having ER+ tumors.

Results

Dicer Antibody Characteristics. We tested 4 different antibodies to human Dicer (Fig.1). Mouse monoclonal antibody 13D6 (Abcam) (8, 9, 11, 18, 19) and 4A6 (Abcam), are made from the same immunogen which is proprietary but contains the central region of Dicer (Clonegene, Hartford, CT, USA) (Fig. 1). Antibody 4A6 had stronger IHC staining compared to 13D6 (not

shown) and was therefore chosen for further analysis. Rabbit polyclonal antibody HPA000694 (Sigma) was chosen due to its extensive use on various tissues by the Human Protein Atlas project (www.proteinatlas.org). HPA000694 targets the PAZ domain (Fig. 1). Rabbit polyclonal antibody ab5818 (Abcam) was chosen because it was one of the few Dicer antibodies with a commercially available blocking peptide when this project was initiated. Ab5818 targets a sequence that is 3' to the PAZ domain and contains the first 5 residues of the Ribonuclease IIIa domain (Fig. 1).

Antibody Comparison of Endogenous Dicer Protein from Breast Cancer Cell Lysates. To examine specificity of the above antibodies, we performed a side-by-side comparison by western blot analysis. T47D and BT549 whole cell lysates were loaded in triplicate on an SDS-PAGE gel and transferred to a PVDF membrane. Following standard western blot procedures as detailed by Mahmood et.al. (20), membranes were blocked, probed with ab5818, 4A6, and HPA000694, washed, probed with the appropriate secondary antibodies, and scanned using a Licor Odyssey Imager. All three antibodies showed higher expression of Dicer in T47D (an ER+ breast cancer cell line) versus BT549 (a basal-like TNBC cell line), as previously reported (15). 4A6 had a strong signal for Dicer with minimal cross-reactivity (fewer lower molecular weight bands) compared to the other antibodies. Both antibodies ab5818 and HPA000694 had more non-specific bands than 4A6, but they also showed clear Dicer signal, with HPA000694 demonstrating the strongest Dicer signal of all three antibodies at the same exposure (Fig. 2).

The synthetic peptide specific for ab5818, when incubated at a concentration of 10 times the primary antibody, effectively blocked Dicer signal of both ER+ (T47D) and TNBC (BT549) lysates Fig. 2).

Antibody Comparison of Overexpressed Dicer. To further test specificity of the antibodies for Dicer we introduced a Dicer cDNA expression vector to cells. HEK293FT cells were transiently

transfected for 48h with either media plus transfection reagent (mock) or the TruORF® pCMV-Entry plasmid containing Human DICER1 (ribonuclease type III, transcript variant 2). Whole cell extracts were loaded in triplicate and western blot analyses with the three antibodies were performed. All antibodies detected higher amounts of Dicer in extract from Dicer overexpressing cells compared to mock transfected cells (Fig. 3), although each antibody demonstrated unique, fairly strong recognition of various non-specific bands. 4A6 had the least amount of non-specific binding, although there was a strong, likely non-specific signal at 50kD. HPA000694 and ab5818 had many non-specific bands, although they both had only one relatively intense non-specific signal at ~145 kD and ~60 kD respectively (Fig. 3.). It should be noted that these bands are assumed to be non-specific because they appear at equal levels in both the mock and Dicer transfected cells and do not correspond with reported molecular weights of documented Dicer isoforms (18).

Antibody Comparison of Endogenous Dicer in Nuclear versus Cytosolic Cellular Fractions. In the literature, the predominant location and activity of Dicer is in the cytoplasm (21, 22); however, nuclear Dicer has been reported (19, 23). Consequently we sought to determine if the antibodies would detect Dicer predominately in the cytoplasmic or nuclear fraction of breast cancer cells. Nuclear and cytosolic protein was prepared from T47D cells grown in 15cm plates at 60-70% confluency using the NE-PER Nuclear and Cytoplasmic Extraction kit (Thermofisher). Immediately following extraction, 90µg of nuclear and cytoplasmic lysates (n=2 biological replicates for each) were loaded in three separate gels and membranes were probed for Dicer using ab5818, 4A6, or HPA000694 (Fig. 4a). Antibodies recognizing human Topoisomerase I (TOPOI), and α-Tubulin, were used to assess the purity of nuclear and cytosolic fractions respectively. All three antibodies showed higher Dicer expression in the cytosolic fractions compared to the nuclear fractions (Fig. 4a).

Antibody Comparison by IHC on FFPE Cells. To evaluate the performance of these antibodies for IHC, T47D cells were formalin fixed, paraffin embedded (FFPE) and stained for Dicer (Fig. 4b). All antibodies were optimized using a standardized HRP IHC protocol (5 min at 125°C heat-induced epitope retrieval in 10mM citrate buffer at pH 6.0). We chose the Vectastain Universal Elite ABC kit for IHC optimization because it detects all IgG classes of primary mouse or rabbit antibodies and has minimal background when used with human tissue (data not shown). Compared to the negative control (no primary antibody; -C, Fig. 4b), antibody ab5818 had strong cytoplasmic staining and 4A6 had weaker cytoplasmic staining, each with minimal staining in the nuclei (Fig. 4b). In contrast, HPA000694 had moderate cytoplasmic staining with strong nuclear staining by IHC which was only very weakly detected in the western blots of the nuclear fractions (Fig 4a, b), suggesting that this antibody binds to a non-specific conformational epitope in the nucleus detected by IHC that is not detected in denatured and reduced protein following SDS-PAGE and western blot analysis.

To further test the specificity of ab5818 we used the blocking peptide specific to ab5818 which effectively blocked Dicer staining in an ER+ clinical sample (Fig. 4c).

Antibody Comparison with miR-200c Manipulation. We and others have previously reported that Dicer is increased by restoring miR-200c to poorly differentiated TNBC and ovarian cancer cells that have very low Dicer and extremely low levels of miR-200c as compared to their more differentiated ER+ counterparts (15, 24). Here we use a doxycyline (DOX)-inducible expression vector for miR-200c in both BT549 TNBC cells and HEY ovarian cancer cells to compare the ability of ab5818 and HPA000694 to detect endogenous Dicer. BT549 cells stably containing the DOX-inducible miR-200c expression vector were treated with or without DOX for 48h, and cells were harvested for RNA/qRT-PCR and FFPE. DOX-induced cells had a 350-fold increase in miR-200c relative to un-induced cells ($p<0.0001$ unpaired t-test) (Fig. 5a left). BT549 -/+ DOX FFPE cell pellets were stained for Dicer using ab5818 and HPA000694. Ab5818 was able

to detect an increase in cytoplasmic Dicer in the DOX-induced cells compared to un-induced cells (Fig. 5a right, top panel). HPA000694 on the other hand detected minimal difference between BT549 cells treated with or without DOX, and had both nuclear and cytoplasmic staining (Fig. 5a right, bottom panel).

HEY ovarian cancer cells stably containing the DOX-inducible expression vector for miR-200c were grown as xenografts in NOD SCID mice treated with or without DOX for 21 days. miR-200c levels in tumors from mice treated with or without DOX were detected by qRT-PCR (16) (Fig. 5b). Dicer expression in three representative xenograft tumors was detected using either ab5818 or HPA000694 (Fig. 5b). Antibody ab5818 had clean moderate cytoplasmic staining with negative nuclei in all three xenografts, with higher intensity staining in the miR-200c expressing tumors compared to the non-miR-200c expressing controls (Fig. 5b, left). Again, HPA000694 had high nuclear and cytoplasmic signal as well as high background in all tumors regardless of miR-200c expression (Fig. 5b right), further confirming the lack of specificity of this antibody by IHC.

Antibody Comparison in Clinical Breast Specimens. We next tested all three antibodies in primary human breast cancer specimens (n=5 ER+ and n=5 TNBC). Although anti-Dicer clone 4A6 was sensitive and specific on immunoblots (Fig. 2-4) it showed weak cytoplasmic staining by IHC at concentrations as high as 1:25, for 1 out of 5 ER+ breast cancers tested, and no staining in TNBCs (Fig 6a, b). Ab5818 showed moderate to strong cytoplasmic staining in all ER+ breast cancers tested, with weaker cytoplasmic staining in TNBC cases (Fig. 6a, b). HPA000694 showed moderate to strong nuclear and cytoplasmic staining in all ER+ breast cancers, and moderate to weak nuclear and cytoplasmic staining in TNBC (Fig. 6a, b). In summary, ab5818 showed the greatest difference between ER+ and ER- (TNBC) and consistently stained the cytoplasm, while HPA000694 showed a less dramatic difference in the

ER+ as compared to TNBC cases, and again appeared to stain both the nuclear and cytoplasmic compartments.

Dicer in Breast Cancer and Normal Breast Tissue. Antibody ab5818 demonstrated both sensitivity and specificity for Dicer by IHC and WB in human breast cancer specimens, cells, and xenografts. Therefore we chose antibody ab5818 to assess Dicer protein by IHC in a larger cohort of ER+ (n=25) and TNBC (n=21) samples. A pathologist (PJ) scored these tissues for Dicer expression in both tumor and adjacent normal tissue compartments. All ER+ tumors showed moderate to strong cytoplasmic staining for Dicer, while all triple-negative tumors showed much weaker cytoplasmic staining compared to the ER+ cohort (Fig. 7a, b). ER+ tumors had an average score of 266.2 for Dicer expression while TNBC had an average Dicer score of 88.9 (Fig. 8a, $p<0.0001$ unpaired t-test). These data were also significant ($p<0.0001$ unpaired t-test) when plotted as either intensity or % positive cells alone (data not shown).

Interestingly, non-involved 'normal' epithelium adjacent to either ER+ or TNBC showed consistently high expression for Dicer (an average score of 285 for ER+ adjacent normal, and 300 for triple-negative adjacent normal). Dicer expression was not significantly different for ER+ tumor vs. adjacent normal ($p = 0.6283$), however, in a paired t-test (n=18 pairs) between triple-negative tumor and its adjacent normal tissue, Dicer was significantly lower in tumor vs. normal $p<0.0001$ (Fig. 8a, b).

Discussion

Reports of Dicer expression in breast cancer in the literature have been variable. By qPCR, a loss of DICER1 expression in TNBC versus normal breast was reported (10), and decreased DICER1 was also associated with TNBC (18, 25). However, a fourth group did not observe a difference between triple-negative and normal DICER1 mRNA (26). Other studies observed that decreased DICER1 levels were associated with breast tumor progression and metastasis

(18), but there is also evidence of elevated levels of DICER1 in tamoxifen-resistant metastatic breast tumors (27). These inconsistencies in Dicer expression even occur within studies where groups have compared Dicer mRNA vs. protein with varying results. For example, Grelier et. al. report that lower DICER1 mRNA expression was associated with lower metastasis-free survival, but that protein levels (using Dicer antibody 13D6) were not predictive of survival, and Martello et. al. report that breast tumors with less intense Dicer staining (using an unspecified Dicer antibody) were associated with metastatic disease, but they did not observe this at the mRNA level (18, 28). It should be noted that in both cases these studies used different cohorts to compare Dicer mRNA and protein. It would be ideal to compare Dicer mRNA and protein within the same cohort, and certain studies have been able to perform qPCR on FFPE tissue, but they claim that Dicer evaluation by IHC was problematic or that the Dicer antibodies were suboptimal, and therefore did not show their IHC results (10, 25).

To help clarify this issue both in the field and for our own research, we tested three Dicer antibodies: Abcam ab5818, Abcam 4A6, and Sigma HPA000694 by western blot and IHC comparing ER+ and TNBC, where we have already observed distinct differences in Dicer protein expression between cell lines (15). All three antibodies detected higher Dicer expression in ER+ breast cancer vs. TNBC by western blot, but had very different staining patterns by IHC (intensity levels, percent positivity, and nuclear/cytoplasmic localization) when the exact same tumor samples were compared. Importantly, antibody HPA000694 detected high levels of Dicer in the nucleus of T47D cells (Fig. 4b), both induced and un-induced BT549 cells (Fig. 5a), and in breast cancer clinical specimens (Fig. 6) by IHC, unlike the other two antibodies.

Small punctate amounts of Dicer have been reported in the nucleus of HeLa (23) and HEK293 (19) cells using confocal microscopy with either a non-commercially available antibody (12B5/4C6), or antibody 13D6, respectively. In the case of the former, digitonin treatment was

needed to wash out the cytoplasm in order to visualize Dicer nuclear staining. Most of the literature using bright field microscopy report cytoplasmic Dicer expression in breast cancer by IHC with the exception of one group who also observed strong nuclear Dicer staining in TNBC by IHC, however the anti-Dicer antibody used for this staining was not completely described (26).

Dicer lacks a canonical nuclear localization signal (NLS) but it has been shown to associate with a component of the nuclear pore complex (23). Other groups have shown that Dicer can shuttle to the nucleus and that the double-stranded RNA binding domain (dsRBD) of Dicer mediates nuclear localization (29, 30). There are three reported isoforms of Dicer (18). The full-length isoform is 1922aa (217kD) and additional isoforms include one lacking the helicase and dsRBD (997aa, 113kD), and one that lacks the helicase and PAZ domain (820aa, 93kD) (Fig. 1). Antibody 13D6 (and therefore 4A6) recognizes all three isoforms of Dicer (18). Ab5818 should also recognize all three isoforms considering its target epitope, and HPA000694 should only recognize the two larger isoforms that contain the PAZ domain, one of which is missing the dsRBD. All of this considered, antibodies 4A6 and ab5818 should recognize the same Dicer isoforms as HPA000694. Therefore, epitope recognition of a particular Dicer isoform does not explain the inconsistency in Dicer staining observed between HPA000694 which is nuclear in breast cancer, compared to the other antibodies in which it is not.

HPA000694 recognizes the PAZ domain, which in eukaryotes is only found in Dicer, Ago, and Piwi subfamilies (31) and the specific sequence that HPA000694 recognizes shows homology with the PAZ domain of piwi-like proteins (ncbi.nlm.nih.gov). Cross-reactivity with piwi-like proteins could possibly explain why HPA000694 shows nuclear as well as cytoplasmic staining in tissues. Piwi-like proteins are important in germline and stem cell maintenance (32), but have also been reported as overexpressed in cancer (33-35). Interestingly, several groups report nuclear staining in addition to cytoplasmic staining of Hiwi (the human homologue of piwil1) by

IHC in various cancers (36, 37) and one group reported that piwil2 nuclear expression was more prevalent in invasive and metastatic breast cancers by IHC (38). Piwi1, piwi2, and piwi4 are in the range of ~97-110kD and the comparison of the 3 different antibodies (Fig. 2) showed bands at that potential size with HPA000694, but the intensity of these bands is faint compared to the intensity of full length Dicer as well as other non-specific bands. This possibility would necessitate further investigation with piwi/Hiwi-specific antibodies. Therefore, at this time we can only speculate that HPA000694 may be binding to a non-specific conformational epitope that is detectable by IHC but not by western blot.

In addition to consistent staining patterns between western blot and IHC, ab5818 had stronger intensity by IHC than that of 4A6 which was weak in our tissue samples at concentrations as high as 1:25 (Fig 6). ab5818 showed clean, distinctive cytoplasmic staining for Dicer that increased with the induction of miR-200c (Fig. 5). The increase in Dicer expression with restoration of miR-200c was consistent with our previous findings in breast cancer cells (15). In addition, ab5818 had a commercially available blocking peptide (representing the immunogen to which the antibody was generated) that when incubated with primary antibody, effectively blocked the detection of Dicer by IHC and western blot (Fig. 2, 4c). Therefore, we decided to use ab5818 for further Dicer IHC analysis of breast cancer specimens. We observed significant differences in Dicer expression between ER+ and TNBC with Dicer being significantly higher in the more well-differentiated ER+ disease ($p<0.0001$), as well as significant differences between TNBC and adjacent non-involved epithelium (Fig. 8a, b), consistent with previous reports (9, 10). These results are also consistent with results observed at the mRNA level in the Curtis Breast Cancer Dataset (17) where DICER1 mRNA was more highly expressed in ER+ vs. ER- breast cancer patients (Fig. 8c).

Dicer expression is currently being examined as a prognostic indicator, which suggests that it may become more clinically relevant. However, the strikingly different results observed

between different Dicer antibodies by IHC highlights the need for further optimization and standardization in order to implement use in a clinical setting. For ongoing research as well, the issue of antibody specificity, and why some antibodies detect nuclear Dicer, while others do not, will need to be further examined to insure proper interpretation of the complex actions of this important enzyme.

Disclosure/Conflict of Interest: Nothing to disclose/ no conflict of interest.

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Titles and Legends to Figures:

Figure 1. Dicer antibody overview. Top) Schematic of Dicer protein, its isoforms, and antibody immunogen sequence homology. HELICASE = Helicase domain, DUF = Domain of unknown function, PAZ = PAZ domain, Rnase III (a and b) = ribonuclease III c terminal domain(s), dsRBD = double stranded RNA binding domain. 93kD and 113kD = isoforms according to Grelier et al. 217kD = full length Dicer protein. Gray line: Clones 13D6 and 4A6, sequence proprietary/unknown corresponding to the central region of Dicer, bold black line: ab5818, residues 1200-1300 including the Rnase IIIa domain, thin black line: HPA000694, residues 869-1062, including the PAZ domain. Bottom) Summary of antibody information.

Figure 2. Antibody comparison of Dicer expression in ER+ vs. triple-negative breast cancer cell lines. All blots were scanned at similar intensity setting. Whole cell lysate of T47D (ER+) and BT549 (triple-negative) breast cancer cell lines probed for Dicer with 4A6, HPA000694, ab5818, and ab5818+10X blocking peptide (Dicer 217kD band highlighted in red box). GAPDH loading control corresponding to the above membrane 35 kD.

Figure 3. Dicer overexpression is well detected by all three antibodies. Whole cell extracts of HEK293FT cells mock transfected or transfected with a Dicer cDNA expression plasmid. Dicer 217kD band highlighted in red box. α-tubulin (50kD) was used as a loading control. All blots were scanned at similar intensity.

Figure 4. Antibody detection of Dicer in nuclear and cytoplasmic fractions and by IHC. A) T47D cell extracts showing Dicer expression in the nuclear versus cytoplasmic fraction with all three antibodies. TOPOI used as a control for nuclear protein. α-tubulin used as a control for cytoplasmic protein. B) T47D cells, FFPE and stained for Dicer with the three antibodies. Bar = 25µm. C) ER+ Breast cancer stained for Dicer with ab5818 alone (left) and with ab5818+10X blocking peptide (right). Bar = 50µm.

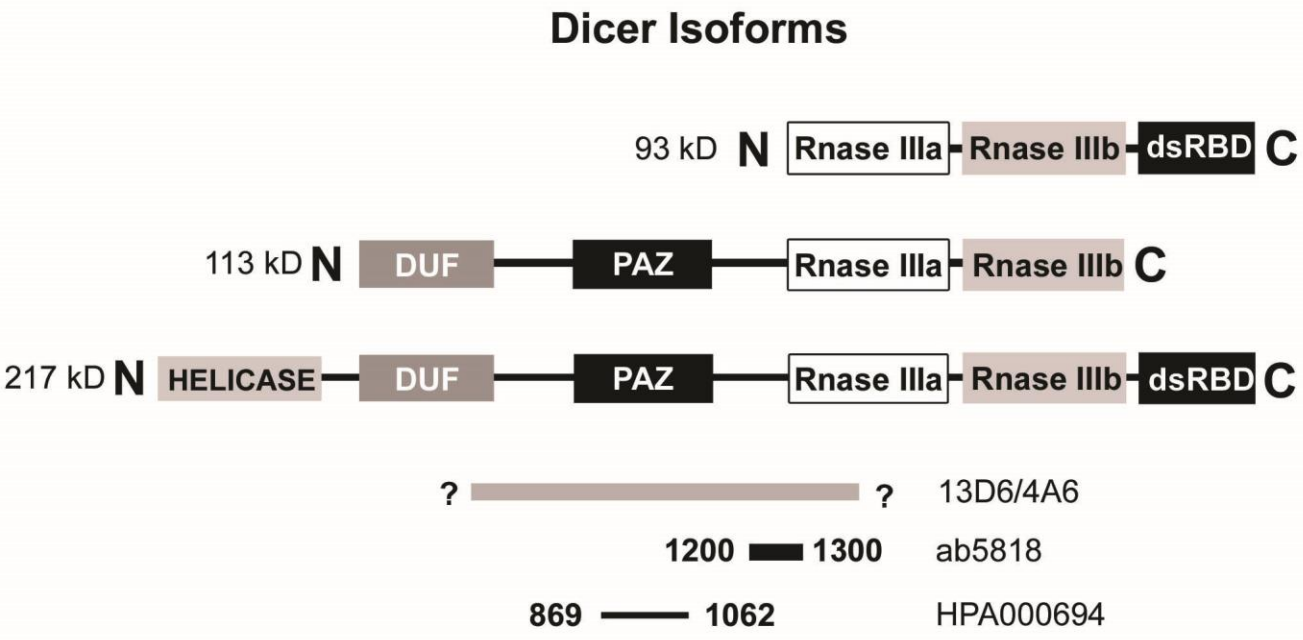
Figure 5. Antibody IHC comparison with miR-200c manipulation in breast cancer cells and ovarian cancer xenografts. BT549-TRIPz-200c cells were untreated (Control) or DOX treated (miR-200c induced) for 48h. A left) relative qRT-PCR for miR-200c. A right) IHC for Dicer using ab5818 and HPA000694, bar = 25µm. B) HEYTGL xenograft tumors with DOX-inducible miR200c. Bottom corners) miR-200c ΔΔct values normalized to U6 for each tumor as detected by qRT-PCR. Untreated (Control) or Dox treated (miR-200c Induced) with 2g/L dox for 21 days, stained for Dicer using ab5818 and HPA000694. Bar = 25µm.

Figure 6. Dicer expression in ER+ versus TNBC clinical specimens as detected by IHC with the three different antibodies. A) ER+ breast cancers each stained with Dicer antibodies 4A6, ab5818, and HPA000694, n=5 bar = 50µm. B) Triple-negative breast cancers each stained with Dicer antibodies 4A6, ab5818, and HPA000694, n=5, bar = 50µm.

Figure 7. Differential expression of Dicer in ER+ vs. TNBC as detected by ab5818 IHC. 20 representative images of A) ER+ and B) TNBC specimens stained for Dicer using ab5818, bar = 50µm.

Figure 8. Quantification of Dicer expression in breast cancer and in adjacent normal breast. A) ER+ and triple-negative breast cancers and adjacent normal (if present in the tissue section) were stained for Dicer using ab5818, and were scored for Dicer expression by a pathologist (IHC score = intensity rating on a scale of 0-4 multiplied by the percent positive). *$p<0.0001$, unpaired t-test. **$p<0.0001$, paired t-test, n=18 pairs. B) Representative images of tumor (T) and adjacent normal tissue (N) defined by blue line in ER+ and TNBC stained for Dicer using ab5818, bar = 50µm. C) Dicer mRNA expression from the Curtis breast cancer data set [17] in ER- vs. ER+ breast cancers, $p<0.0001$, unpaired t-test.

Figure 1



Vendor	Antibody/Clone	Clonality	Subclass	Sequence
Clonegene	13D6	monoclonal	IgG2a	Central Region
Clonegene	4A6	monoclonal	IgG1	Central Region
Abcam	ab5818	polyclonal	IgG	1200-1300
Sigma	HPA000694	polyclonal	IgG	869-1062

Figure 2

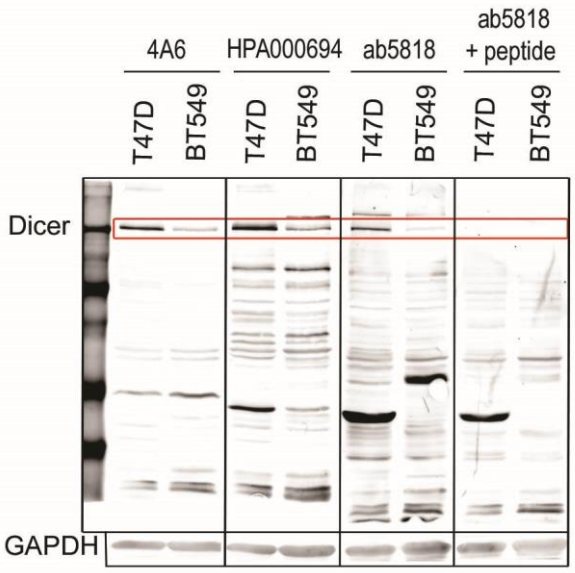


Figure 3

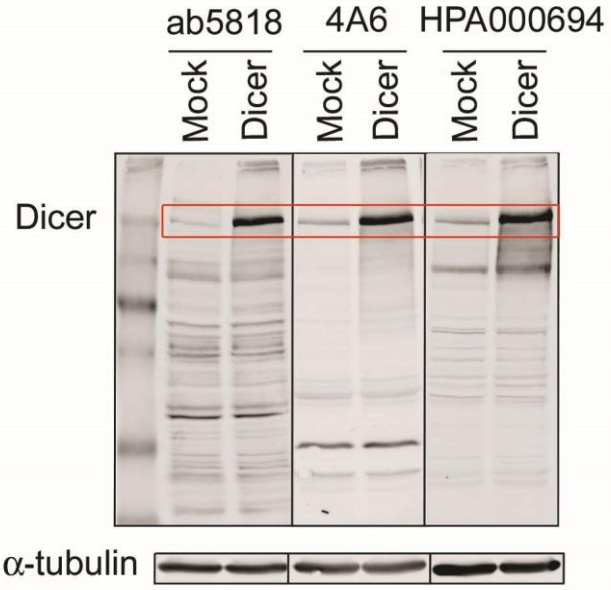


Figure 4

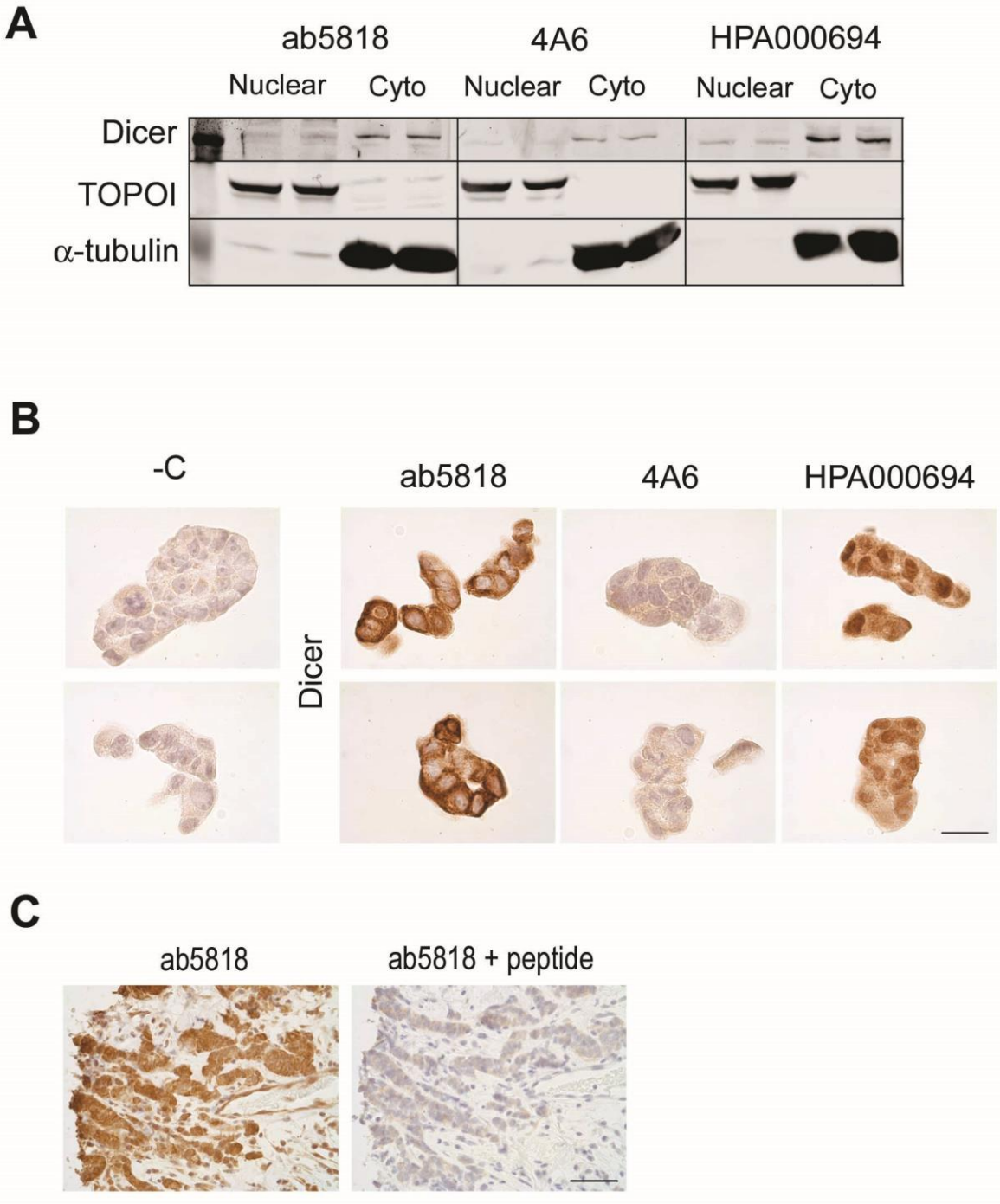

A
ab5818
4A6
HPA000694
Nuclear
Cyto
Nuclear
Cyto
Nuclear
Cyto
Dicer
TOPOI
α-tubulin
B
-C
ab5818
4A6
HPA000694
Dicer
C
ab5818
ab5818 + peptide

Figure 5

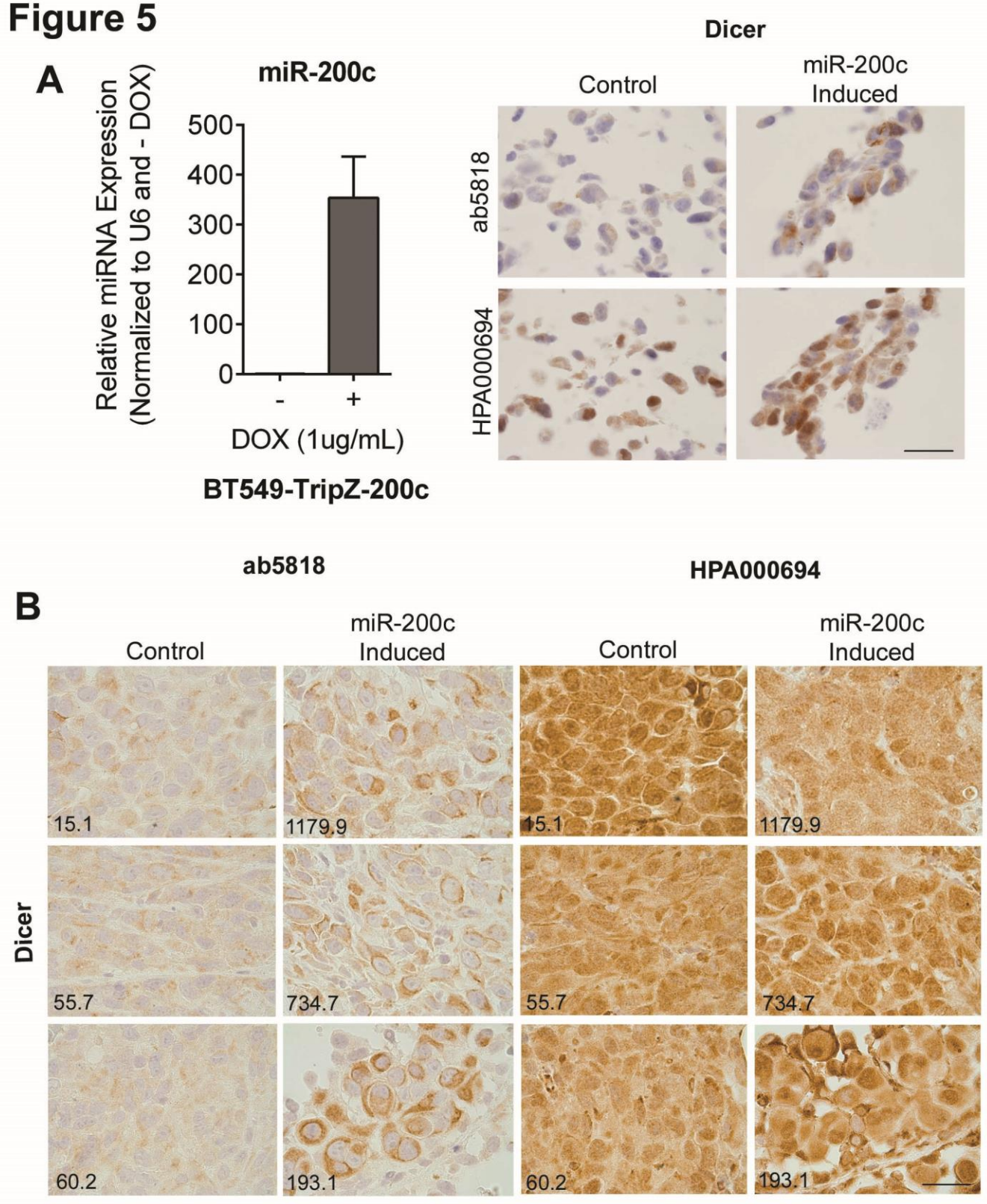

A
miR-200c
Relative miRNA Expression
(Normalized to U6 and - DOX)
500
400
300
200
100
0
-
+
DOX (1ug/mL)
BT549-TripZ-200c
Dicer
Control
miR-200c
Induced
ab5818
HPA000694
B
ab5818
HPA000694
Control
miR-200c
Induced
Dicer
15.1
1179.9
55.7
734.7
60.2
193.1

Figure 6

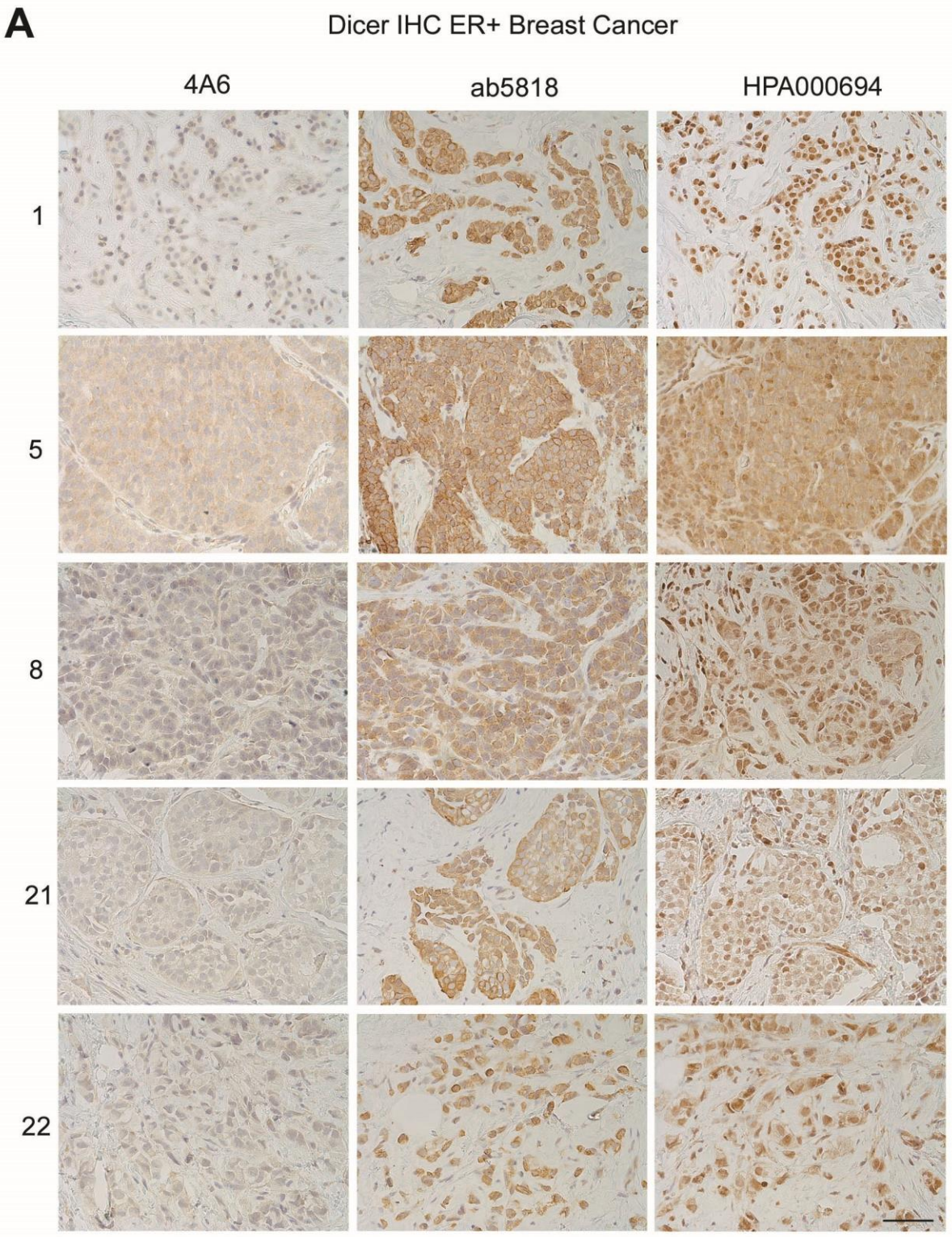


Figure 6

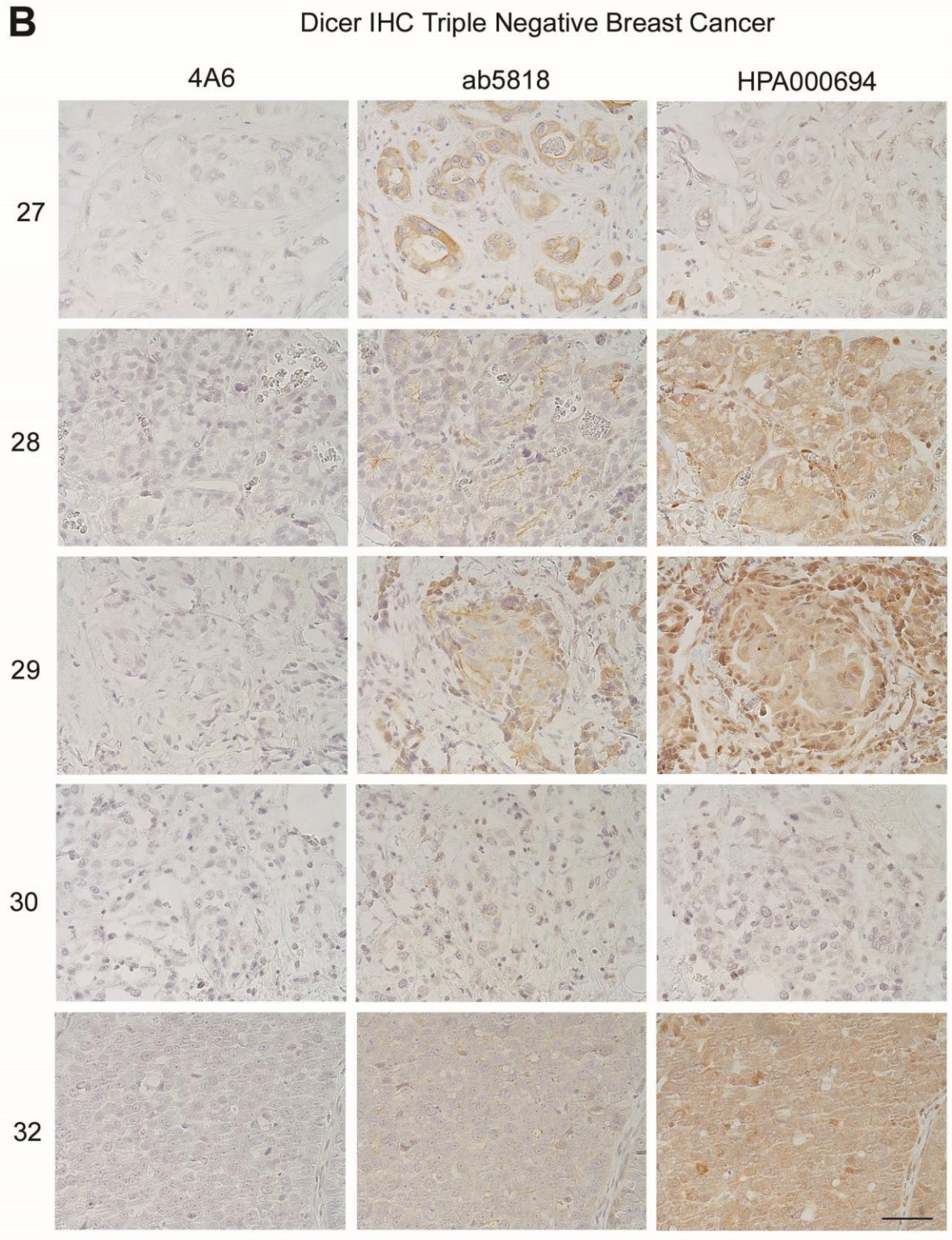

B
Dicer IHC Triple Negative Breast Cancer
4A6
ab5818
HPA000694
27
28
29
30
32

Figure 7

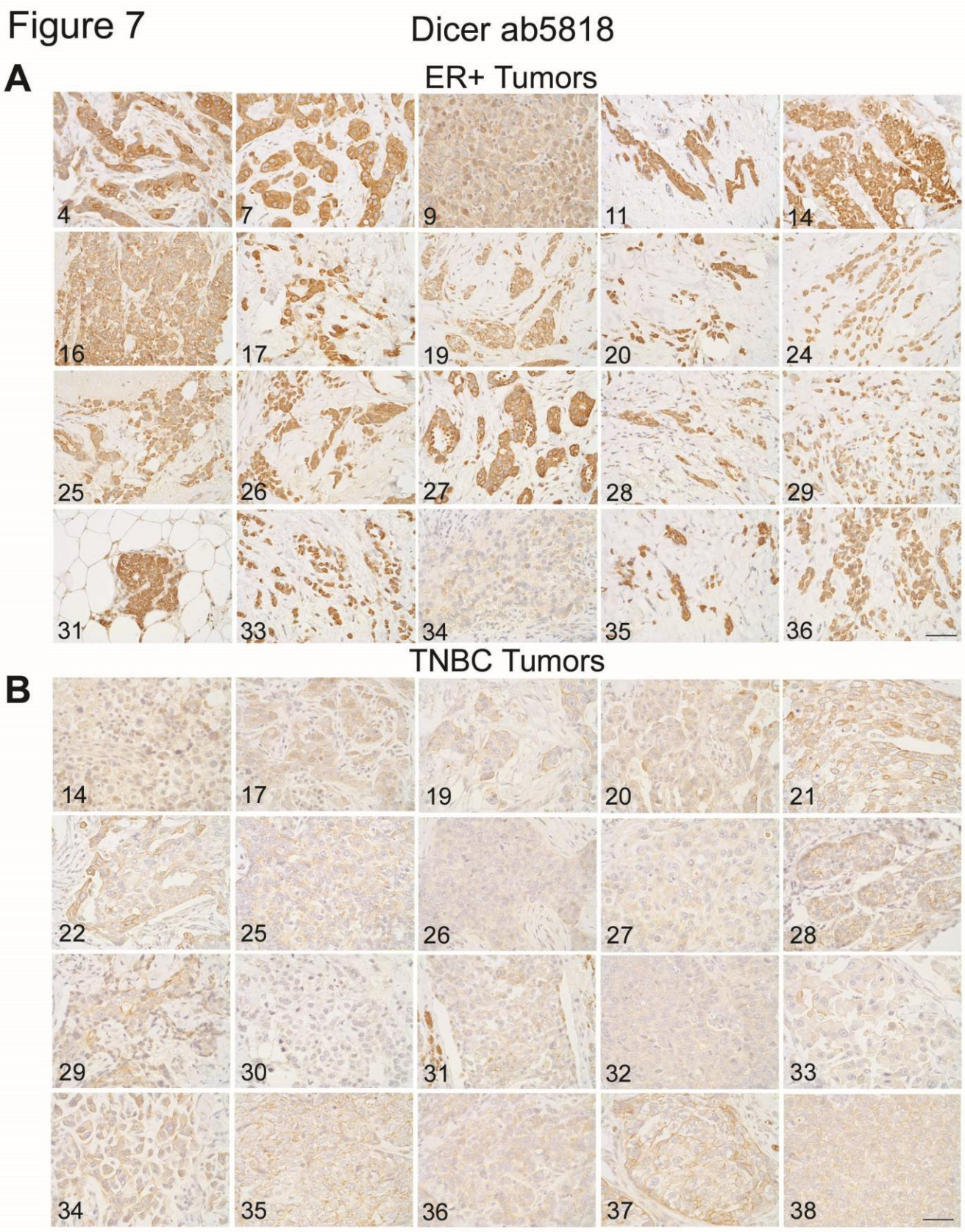
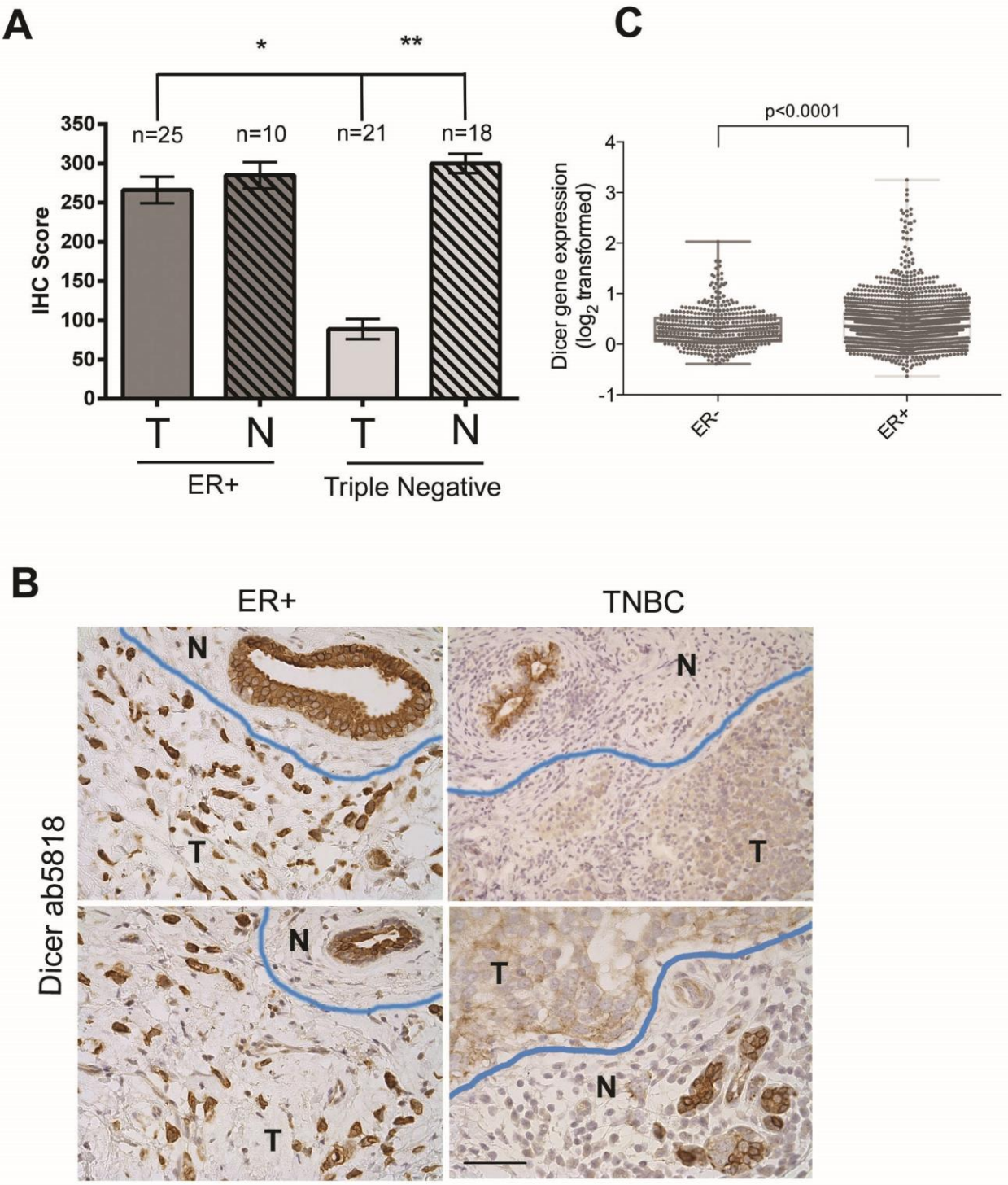

Dicer ab5818
A
ER+ Tumors
4
7
9
11
14
16
17
19
20
24
25
26
27
28
29
31
33
34
35
36
TNBC Tumors
B
14
17
19
20
21
22
25
26
27
28
29
30
31
32
33
34
35
36
37
38

Figure 8


A
*
**
n=25
n=10
n=21
n=18
IHC Score
350
300
250
200
150
100
50
0
T
N
T
N
ER+
Triple Negative
C
p<0.0001
Dicer gene expression
(log2 transformed)
4
3
2
1
0
-1
ER-
ER+
B
ER+
TNBC
Dicer ab5818
N
T
N
T
N
T
T
N

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Tumor and Stem Cell Biology

Cancer Research

A TDO2-AhR Signaling Axis Facilitates Anoikis Resistance and Metastasis in Triple-Negative Breast Cancer

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Abstract

The ability of a cancer cell to develop resistance to anoikis, a programmed cell death process triggered by substratum detachment, is a critical step in the metastatic cascade. Triple-negative breast cancers (TNBC) exhibit higher rates of metastasis after diagnosis, relative to estrogen-positive breast cancers, but while TNBC cells are relatively more resistant to anoikis, the mechanisms involved are unclear. Through gene expression and metabolomic profiling of TNBC cells in forced suspension culture, we identified a molecular pathway critical for anchorage-independent cell survival. TNBC cells in suspension upregulated multiple genes in the kynurenine pathway of tryptophan catabolism, including the enzyme tryptophan 2,3-dioxygenase (TDO2), in an NF-κB–dependent manner. Kynurenine production mediated by TDO2 in TNBC cells was sufficient to activate aryl hydrocarbon receptor (AhR), an endogenous kynurenine receptor. Notably, pharmacologic inhibition or genetic attenuation of TDO2 or AhR increased cellular sensitivity to anoikis, and also reduced proliferation, migration, and invasion of TNBC cells. *In vivo*, TDO2 inhibitor–treated TNBC cells inhibited colonization of the lung, suggesting that TDO2 enhanced metastatic capacity. In clinical specimens of TNBC, elevated expression of TDO2 was associated with increased disease grade, estrogen receptor–negative status, and shorter overall survival. Our results define an NF-κB–regulated signaling axis that promotes anoikis resistance, suggest functional connections with inflammatory modulation by the kynurenine pathway, and highlight TDO2 as an attractive target for treatment of this aggressive breast cancer subtype. *Cancer Res; 75(21); 4651–64. ©2015 AACR.*

Introduction

The vast majority of breast cancer deaths are caused by complications from metastases (1–3). A high rate of metastasis is characteristic of triple-negative breast cancers (TNBC), which lack expression of estrogen receptor (ER), progesterone receptor (PR), and HER2 amplification, and thus do not respond to current endocrine therapies or HER2-targeted therapies (4). Due in part to the absence of effective targeted therapies, but likely also to inherent properties of this subtype, patients with metastatic TNBC have a poor prognosis with a median survival of 13 months (5). Thus, identification of new targeted therapies that inhibit or slow metastasis is of critical importance to improve the prognosis of women with TNBC.

Metastasis is a complex process with multiple steps, including detachment of cancer cells from the primary tumor, invasion through local tissue, intravasation into the vasculature and lymphatics, survival while in transit, extravasation, and colonization of secondary sites (6). Normal epithelial cells are programmed to undergo apoptosis if they become detached from the basement membrane, a process termed anoikis (7). Anoikis resistance is thought to facilitate survival of tumor cells that detach from the primary tumor and thereby facilitate metastasis (8, 9). Multiple mechanisms of anoikis resistance have been identified, including deregulation of integrin expression and aberrant activation of prosurvival pathways (as reviewed in ref. 9), as well as altered metabolism (10, 11). However, the present study is the first to globally profile gene expression alterations in forced suspension culture with the goal of identifying targetable pathways important for survival in suspension. This objective screening approach identified multiple components of the kynurenine pathway of tryptophan catabolism and the aryl hydrocarbon receptor (AhR), which is activated by kynurenine (12), as being upregulated by TNBC in suspension culture.

Altered tryptophan metabolism and increased secretion of tryptophan metabolites by solid tumors, including in breast cancer, have long been recognized (13, 14). The essential amino acid tryptophan is required for protein synthesis and is a precursor for the formation of multiple signaling molecules including serotonin (15). The majority of tryptophan catabolism occurs via the kynurenine pathway, leading to synthesis of NAD^+ along with intermediate products, including quinolinic acid and

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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kynurenine (Kyn; ref. 16). The first step of the kynurenine pathway can be catalyzed by indoleamine 2,3-dioxygenase 1 (IDO1), IDO2, or tryptophan 2,3-dioxygenase (TDO2; refs. 17–19). IDO1 is expressed in tissues throughout the body, whereas TDO2 is predominantly expressed in the liver (20). Much research on the kynurenine pathway in cancer has focused on the ability of Kyn to decrease immune surveillance (as reviewed in ref. 21), but in glioma, Kyn, acting in both a paracrine and an autocrine fashion, suppressed antitumor immune responses and promoted tumor cell survival and motility. Kyn was demonstrated to serve as an endogenous ligand to activate AhR in both immune cells and the tumor cells themselves (12).

AhR is a member of the basic-helix-loop-helix (bHLH) Per-ARNT-Sim (PAS) superfamily of transcription factors best known for its activation by xenobiotics such as polycyclic aromatic hydrocarbons (PAH; refs. 22, 23). PAH binding leads to transcription of AhR target genes, including drug-metabolizing enzymes such as *CYP1A1* and *CYP1B1* (24). Increased expression of AhR and its target genes has been found in several cancer types, including lung, cervical, ovarian, and breast (16). AhR is required for normal mammary gland development (25, 26), and AhR overexpression increased migration, invasion, and proliferation of immortalized mammary epithelial cells (27). AhR knockdown in MDA-MB-231 TNBC cells decreased expression of genes involved in these same processes (28). Interestingly, increased AhR activity in mouse hepatoma cells grown in suspension was observed nearly 20 years ago (29); however, neither the mechanism of activation nor the functional significance was tested.

Using global expression analysis and metabolomic profiling of TNBC cells in forced suspension, we identify a novel TDO2-AhR signaling axis, mechanistically dependent on NF-κB, which promotes anoikis resistance, as well as migratory and invasive capacity. Indeed, we found that pharmacologic inhibition or knockdown of TDO2 or AhR decreased anchorage-independent growth and invasive capacity *in vitro* and TDO2 inhibition decreased lung metastasis in a TNBC preclinical model. Collectively, these data and the fact that *TDO2* expression confers a shorter overall survival in breast cancer patients suggest that TDO2 inhibition may be a rational targeted therapy to reduce TNBC metastasis and resultant mortality.

Materials and Methods

Cell culture and treatments

All cell lines were authenticated by short tandem repeat analysis and tested negative for mycoplasma in July of 2014. SUM159PT cells were purchased from the University of Colorado Cancer Center Tissue Culture Core in August of 2013 and were grown in Ham's F-12 with 5% FBS, penicillin/streptomycin, hydrocortisone, insulin, HEPES, and L-glutamine supplementation. MDA-MB-231 (MDA-231) cells were purchased from the ATCC in August of 2008 and were grown in minimum essential media with 5% FBS, penicillin/streptomycin, HEPES, L-glutamine, non-essential amino acids, and insulin supplementation. BT549 cells, purchased from the ATCC in 2008, were grown in RPMI Medium 1640 with 10% FBS, penicillin/streptomycin, and insulin. MCF7 and T47D cells were purchased from the ATCC and were grown in DMEM with 10% FBS and 2 mmol/L L-glutamine.

Forced suspension culture

Poly-2-hydroxyethyl methacrylate (poly-HEMA, from Sigma-Aldrich) was reconstituted in 95% ethanol to 12 mg/mL. Ethanol was allowed to evaporate overnight, and plates were washed with PBS prior to use.

Kynurenine high performance liquid chromatography

Experimental samples were generated by plating 500,000 cells in 2 mL culture media in a 6-well plate in triplicate. After 48 hours, media were collected and protein was precipitated using trichloroacetic acid at a final concentration of 7%, samples were centrifuged, and the supernatant was analyzed on an Agilent 1260 high performance liquid chromatography (HPLC) with a Kinetex BiPhenyl column (particle size 2.7 μm, 4 mm internal diameter by 150 mm; Phenomenex, catalog no. 00F-4622-E0). Kynurenine was eluted using a binary gradient consisting of 0.2% formic acid in water (mobile phase A) and 0.2% formic acid in acetonitrile (mobile phase B). The separation gradient was 5% to 60% B over 6 minutes at a flow rate of 0.75 mL/min, and the kynurenine peak was monitored at 360 nm. To quantify kynurenine in the samples, peak areas were compared with a serial dilution of L-kynurenine (Sigma; catalog no. K8625) in tissue culture media, which was prepared with the same precipitation method as the samples. In brief, 100 μL of standard or supernatant was injected onto the system, and peak area was calculated using Agilent ChemStation software.

Cellular assays and reagents

Cells were treated with 680C91 and CH223191 (TOCRIS Bioscience) prepared in dimethyl sulfoxide (DMSO). Alpha-naphthoflavone (Sigma-Aldrich) was diluted in methanol:ethyl acetate (3:1), and 1-D,L-methyl-tryptophan and crystalline L-kynurenine (Sigma-Aldrich) were prepared in 0.25N HCl. PS 1145 (TOCRIS Bioscience) was prepared in DMSO. IL1β and TNFα were obtained from eBioscience.

Cellular fractionation

Cellular fractionation was performed using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce Biotechnology) as per the manufacturer's instructions.

Quantitative RT-PCR

Total RNA was isolated using the RNeasy Mini Plus Kit (QIAGEN) according to the manufacturer's instructions. cDNA was synthesized with the Applied Biosystems reverse transcription Kit. qRT-PCR was performed in an ABI 7600 FAST thermal cycler using ABsolute Blue qPCR SYBR Green Low ROX Mix (Thermo Scientific). All fold-change data were normalized to β-actin. All experiments were performed in biologic triplicate. For primer sequences, see Supplementary Methods.

Immunoblotting

Whole-cell protein extracts (50 μg) were denatured, separated on SDS-PAGE gels, and transferred to polyvinylidene fluoride membranes. After blocking in 3% BSA in Tris-buffered saline–Tween, membranes were probed overnight at 4°C. Primary antibodies used include AhR (#13790; 1:1,000 dilution; Cell Signaling Technology), KYNU (H00008942-B01; 1:1,000 dilution; NOVUS Biologicals), TDO2 (H00006999-B01P; 1:1,000 dilution; Abnova), TOPO1 (C-21; 1:100 dilution; Santa Cruz Biotechnology, Inc.), and α-tubulin (clone B-5-1-2; 1:30,000 dilution; Sigma Aldrich). Following secondary antibody incubation, results were detected using Western Lighting Chemiluminescence Reagent Plus (Perkin Elmer). Densitometry quantification was performed using Image Studio Lite

Version 3.1 and reported as a ratio compared with α-tubulin as a loading control.

Immunohistochemistry

Formalin-fixed, paraffin-embedded (FFPE) tissue sections (5 μm) were heat immobilized onto glass slides and deparaffinized in a series of xylenes and graded ethanols. Antigens were heat retrieved in 10 mmol/L Tris, 1 mmol/L EDTA, pH 9.0 solution, and blocked for endogenous peroxidase followed by 10% normal goat serum prior to antibody incubation. Tris-buffered saline with 0.05% Tween (TBST) was used for all washes. TDO2 antibody (Abnova; # H00006999-B01P) was incubated overnight at room temperature at 1:200 in TBST, and AhR antibody (CST #13790) was incubated for 1 hour at room temperature at 1:50 in TBST. Envision polymer (Dako) was used for detection, followed by 3,3′-diaminobenzidine (Dako), and slides were counterstained with dilute hematoxylin.

Gene expression array analysis

BT549 cells were grown in either attached conditions or forced-suspension conditions on poly-HEMA–coated plates in quadruplicate for 24 hours. RNA was harvested at 24 hours using Trizol method, and hybridized onto Affymetrix Human Gene 1.0ST arrays at the University of Colorado Denver Genomics and Microarray Core, following the manufacturer's instruction.

Microarray analysis was performed using Partek Genomics Suite (Partek, Inc.) and Ingenuity Pathway Analysis software (Qiagen, Inc.). One-way ANOVA analysis was performed to determine differentially expressed genes between the two treatment groups (attached vs. suspended). Fold-change cutoff was 1.5, and significance cutoff was $P < 0.05$. Significantly differentially expressed genes were imported to Ingenuity for pathway analysis, including identification of altered canonical pathways.

shRNA experiments

High-titer shRNA lentiviral transduction particle suspensions were obtained from the Functional Genomics Facility at the University of Colorado (Boulder, CO). BT549 or MDA-MB-231 cells were plated at 50% confluence in 60-mm tissue culture–treated dishes in 4 mL of media. Twenty-four hours after plating, 100 μL of the lentiviral suspension and 8 μg/mL polybrene (Sigma-Aldrich) were added to the tissue culture media. After 24 hours, viral media were replaced with regular tissue culture media and cells were incubated for an additional 24 hours before the commencement of puromycin selection (1 μg/mL). Knockdowns were confirmed by Western blot, and all experiments were done within three passages of puromycin selection. For shRNA sequences, see Supplementary Methods.

Luciferase reporter activity

The AhR luciferase reporter, generously provided by Dr. Michael Denison (University of California-Davis), and NF-κB luciferase reporter, generously provided by Dr. Rebecca Schweppe (University of Colorado-Anschutz Medical Campus), were transiently transfected along with SV40 Renilla in TNBC cells using Lipofectamine (Life Technologies). Transfected cells were then plated in a 24-well plate at a density of 5×10^5 cells per well in either a control or poly-HEMA–coated well and incubated at 37°C for 24 hours. Following incubation, reporter activation was determined using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. Briefly, cells were lysed for 15 minutes at room temperature using 1× passive lysis buffer. Lysed cells were collected and centrifuged at 14,000 rpm for 15 minutes at 4°C to eliminate cell debris. The supernatant was used immediately or diluted with 1× passive lysis buffer for determination of luciferase activity. For analysis, AhR or NF-κB reporter activity was normalized to SV40 reporter activity to control for differences in transfection efficiency.

Measurement of anoikis

Soft-agar assays were performed in 0.5% bottom and 0.25% top-layer agar (Difco Agar Noble; BD Biosciences). Media with treatment were refreshed every 4 days.

Caspase-3/7 activity was measured using a Caspase-Glo 3/7 Assay Kit (Promega) according to the manufacturer's protocol. Briefly, 8×10^4 TNBC cells were plated in a clear bottom, white-walled 96-well plate coated with poly-HEMA. The cells were incubated for 48 hours at 37°C. Following incubation, each well was mixed with Caspase-Glo 3/7 reagent in equal volume to the culture medium. The 96-well plate was then covered in foil, shook for 30 seconds, and incubated at room temperature for 30 minutes. Luminescence was determined by luminometer, measuring luminescence after 1 second.

In vivo metastasis experiment

Tail-vein injection experiments were approved by the University of Colorado Institutional Animal Care and Use Committee [IACUC protocol—83612(10)1E]. All animal experiments were conducted in accordance with the NIH Guidelines of Care and Use of Laboratory Animals. Prior to tail-vein injection, luciferase-expressing MDA-MB-231 cells were grown in forced suspension conditions for 48 hours in the presence of either vehicle control or 10 μmol/L 680C91. A total of 250,000 viable cells, as determined by trypan blue staining, were then injected into the tail vein of NOD.CB17-Prkdc^{scid}/J (NOD/SCID) mice (The Jackson Laboratory). Following injection, luminescence was monitored every 7 days for a total of 4 weeks. At the completion of this experiment, lungs of 5 mice from each group were imaged by IVIS *ex vivo*. Lungs from the remaining 5 mice per group were formalin-fixed and paraffin-embedded for IHC.

Proliferation, migration, and invasion

Proliferation assays were performed using the Incucyte ZOOM live-cell imaging system (Essen BioSciences). Migration scratch wound assays were performed per the manufacturer's instructions and scanned with the Incucyte ZOOM apparatus (Essen BioSciences). Trans-well invasion assays were performed with BD BioCoat Matrigel Invasion Chambers (BD Biosciences) per the manufacturer's protocol.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5. Student *t* test, ANOVA with Tukey post-hoc test, and two-way ANOVA with Bonferroni multiple comparison test were used as noted. *P* values are denoted as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant.

Results

Multiple enzymes of the kynurenine pathway are upregulated in detached TNBC cell lines

To model loss of attachment *in vitro*, we grew breast cancer cells on tissue culture plates coated with poly(2-hydroxyethyl

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methacrylate) (poly-HEMA). We previously demonstrated that TNBC cells grown in forced suspension upregulate proteins involved in anoikis resistance and motility (30, 31). To identify additional pathways responsible for resistance to anoikis in an unbiased, global fashion, expression profiling was performed on the TNBC cell line BT549 grown in standard attached conditions for 24 hours as compared with the same number of cells grown in forced suspension for 24 hours in quadruplicate. In total, we identified 367 genes that change more than 1.5-fold in a statistically significant manner ($P < 0.05$, log-rank test). Of these genes, 217 (59%) were upregulated and 150 (41%) were downregulated in suspended cells (Supplementary Fig. S1A). Ingenuity Pathway Analysis of these data revealed two related pathways strongly upregulated in suspension: tryptophan catabolism and AhR signaling (Supplementary Fig. S1B and S1C).

Figure 1A shows unsupervised hierarchical clustering of the top 35 genes significantly upregulated by at least 2-fold in suspended compared with attached conditions ($P < 0.05$, log-rank test). Among the genes most highly upregulated in suspension were two enzymes involved in the kynurenine pathway, which converts tryptophan to NAD^+. The rate-limiting enzyme tryptophan 2,3-dioxygenase (*TDO2*) was the most highly upregulated gene in suspension, and the downstream enzyme kynureninase (*KYNU*) was also among the top fold-changing genes (Fig. 1A). To confirm our gene array data, we performed qRT-PCR for *TDO2* and *KYNU* in multiple breast cancer cell lines, including both luminal (ER^+) and TNBC (ER^-) lines, after 24 hours in suspension (Fig. 1B and C). In all three TNBC lines tested, *TDO2* and *KYNU* were significantly increased in suspension compared with attached culture. In the two ER^+ breast cancer cell lines tested, expression of these genes trended slightly higher in suspension, but this change was not significant.

Western blot analysis of whole-cell extracts also demonstrated an increase in TDO2 and KYNU proteins in TNBC cell lines (MDA-231, BT549, and SUM159) grown in suspension for 24 hours (Fig. 1D; and Supplementary Fig. S3A). The increase in TDO2 protein was confirmed by IHC in BT549 cells grown in suspension for 48 hours compared with cells grown in the attached condition (Fig. 1E).

Global metabolomic profiling of intracellular and secreted metabolites from BT549 cells grown in standard attached conditions or in forced suspension for 24 hours was also performed. Two intermediate products of the kynurenine pathway, Kyn and formylkynurenine, were the intracellular metabolites with the highest fold-change increase in suspension. Among secreted metabolites, kynurenine had the third-highest fold-change increase (Supplementary Fig. S2). Together with the gene expression data, this demonstrates that the kynurenine pathway is strongly upregulated in TNBC cells upon loss of attachment.

Using HPLC to verify the metabolomic profiling data, we found that secreted Kyn levels were more than 2-fold higher in conditioned media from BT549 cells in forced suspension for 48 hours than in media from the same number of cells in the attached condition (Fig. 1F). Furthermore, addition of the TDO2-specific inhibitor 680C91 to cells in suspension completely prevented the increase in secreted Kyn, demonstrating that increased secretion of Kyn in TNBC cells in suspension is dependent on TDO2 activity (Fig. 1F). *TDO2* mRNA was expressed at a higher copy number at baseline than *IDO1* in two of the three TNBC cell lines tested, and *TDO2* expression increased more strongly in suspension than *IDO1* (Supplementary Fig. S3B).

Aryl hydrocarbon receptor expression and activity are increased in suspension

In addition to the components of the kynurenine pathway, *AhR* and AhR-regulated genes were also identified among the genes most highly upregulated by TNBC cells in suspension (Fig. 1A and Supplementary Fig. S4). Increased *AhR* mRNA expression in suspended compared with attached TNBC cells was confirmed by qRT-PCR. Similar to *TDO2* and *KYNU*, *AhR* was not significantly increased in ER^+ cell lines in suspension (Fig. 2A). Furthermore, AhR protein was increased in all three TNBC cell lines tested following 24 hours in suspension as tested by Western blot (Fig. 1D; Supplementary Fig. S3A), and in BT549 cells grown in suspension for 48 hours as measured by IHC (Fig. 2C). In attached SUM159 cells, while AhR protein was found in both the cytoplasmic and nuclear fractions, after 48 hours in forced suspension, AhR protein was almost exclusively nuclear (Fig. 2B). Because AhR nuclear translocation and transcriptional activity are known to be ligand-mediated (32), the movement of AhR to the nucleus in suspension suggests that the receptor is ligand-bound and transcriptionally active.

We assessed AhR transcriptional activity using a luciferase reporter containing six dioxin response elements (DRE), the consensus AhR-binding site. In BT549 and MDA-231 cells, luciferase activity was upregulated more than 2-fold in suspended compared with attached cells (Fig. 2D). The AhR antagonist α-naphthoflavone inhibited the suspension-mediated increase in luciferase activity, demonstrating specificity of the reporter construct for AhR activity (Fig. 2D; Supplementary Fig. S3C).

TDO2-mediated kynurenine production activates AhR

Kyn is an endogenous ligand for AhR (12, 33–35), suggesting a potential relationship between increased kynurenine pathway enzyme expression and increased AhR activity in TNBC cells in suspension. First, we tested whether inhibition of TDO2 and consequent Kyn production could prevent AhR activation in suspended TNBC cells. Treatment with 10 μmol/L 680C91, which prevented the suspension-induced increase in Kyn, significantly decreased AhR activity in BT549 and MDA-MB-231 cells in suspension (Fig. 2E; Supplementary Fig. S3D). Furthermore, the suspension-mediated increase in AhR target genes *CYP1A1* and *CYP1B1* was abrogated by both the AhR inhibitor CH-223191 and the TDO2 inhibitor 680C91 (Fig. 2F), linking TDO2 activity to AhR transcriptional activity. Addition of 100 μmol/L exogenous Kyn also resulted in a significant increase in AhR luciferase reporter activity in TNBC cells in both attached and suspended culture conditions (Fig. 2G), demonstrating that Kyn is able to activate AhR in TNBC cells.

Inhibition of TDO2 and AhR decreases TNBC anoikis resistance, proliferation, migration, and invasion

Because TDO2 and AhR were upregulated by TNBC cells in suspension, we next tested their functional importance by assessing whether pharmacologic inhibition or genetic knockdown could reduce anchorage-independent growth in soft agar or increase sensitivity of TNBC cells to anoikis in forced suspension culture. BT549 and SUM159PT cells were pretreated in the attached condition with vehicle, the TDO2 inhibitor 680C91, or the AhR antagonist CH-223191 for 24 hours. Then an equal number of cells were plated in soft agar in the continued presence of treatment. Both 680C91 and CH-223191 significantly decreased anchorage-independent growth of BT549 and SUM159 cells in soft

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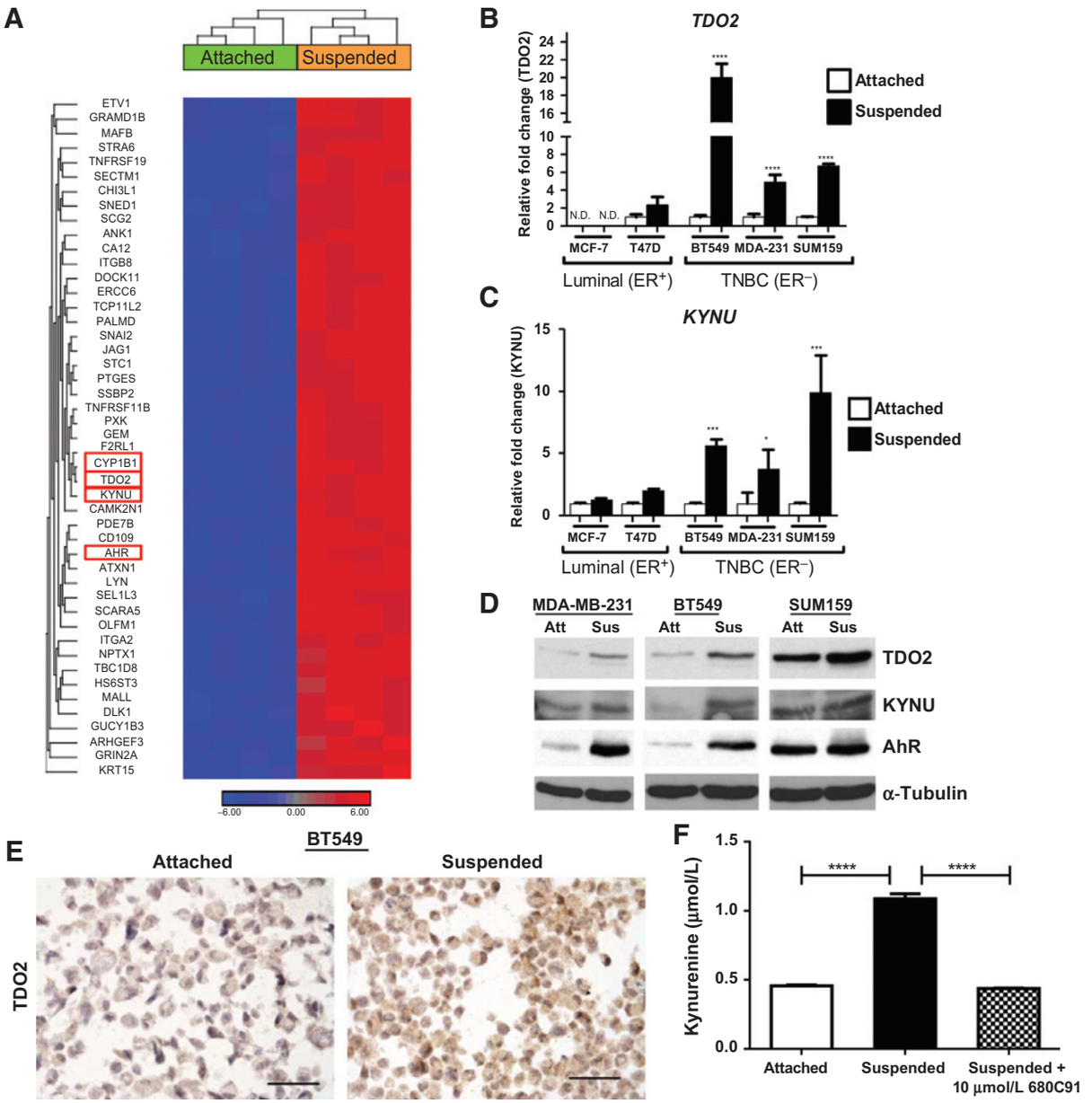

Figure 1.

Kynurenine pathway components and activity are increased in TNBC cells in suspension and suppressed by TDO2 inhibition. A, BT549 cells were grown in either attached or forced-suspension conditions on poly-HEMA–coated plates in biologic quadruplicate for 24 hours. RNA was harvested at 24 hours and hybridized onto Affymetrix Human Gene 1.0ST array. Gene expression profile analysis identified genes significantly upregulated in suspension culture ($P < 0.05$). Those involved in tryptophan metabolism and AhR signaling are highlighted in red. B and C, relative TDO2 (B) and KYNU (C) mRNA levels were determined by qRT-PCR in luminal and TNBC cell lines grown in attached or suspended culture for 24 hours. D, Western blot for TDO2, KYNU, and AhR protein in three TNBC cell lines following 24 hours of attached or suspended culture. E, TDO2 protein detected by IHC in BT549 cell pellets grown in attached or suspended culture for 48 hours. Bar, 50 μm. F, secreted kynurenine levels, as measured by HPLC, from BT549 cells grown for 48 hours in the attached versus suspended conditions or suspended plus 10 μmol/L 680C91 (a specific TDO2 inhibitor). *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$ by ANOVA with Bonferroni multiple comparison test.

agar (Fig. 3A). To test the effect of Kyn on anoikis resistance, we treated cells in forced suspension culture for 48 hours with Kyn and found that this significantly decreased apoptosis as measured by cleaved caspase activity compared with vehicle control treatment (Fig. 3B). We then performed knockdown of TDO2 and AhR using two shRNA constructs each, and decreased protein expression was confirmed by Western blot (Fig. 3C). Knockdown of either TDO2 or AhR also significantly decreased growth of BT549 cells in soft agar (Fig. 3D). Knockdown of either TDO2 or AhR also significantly increased apoptosis in BT549 and MDA-MB-231 cells grown in suspension for 48 hours (Fig. 3E), demonstrating that TDO2 and AhR promote survival in anchorage-independent conditions.

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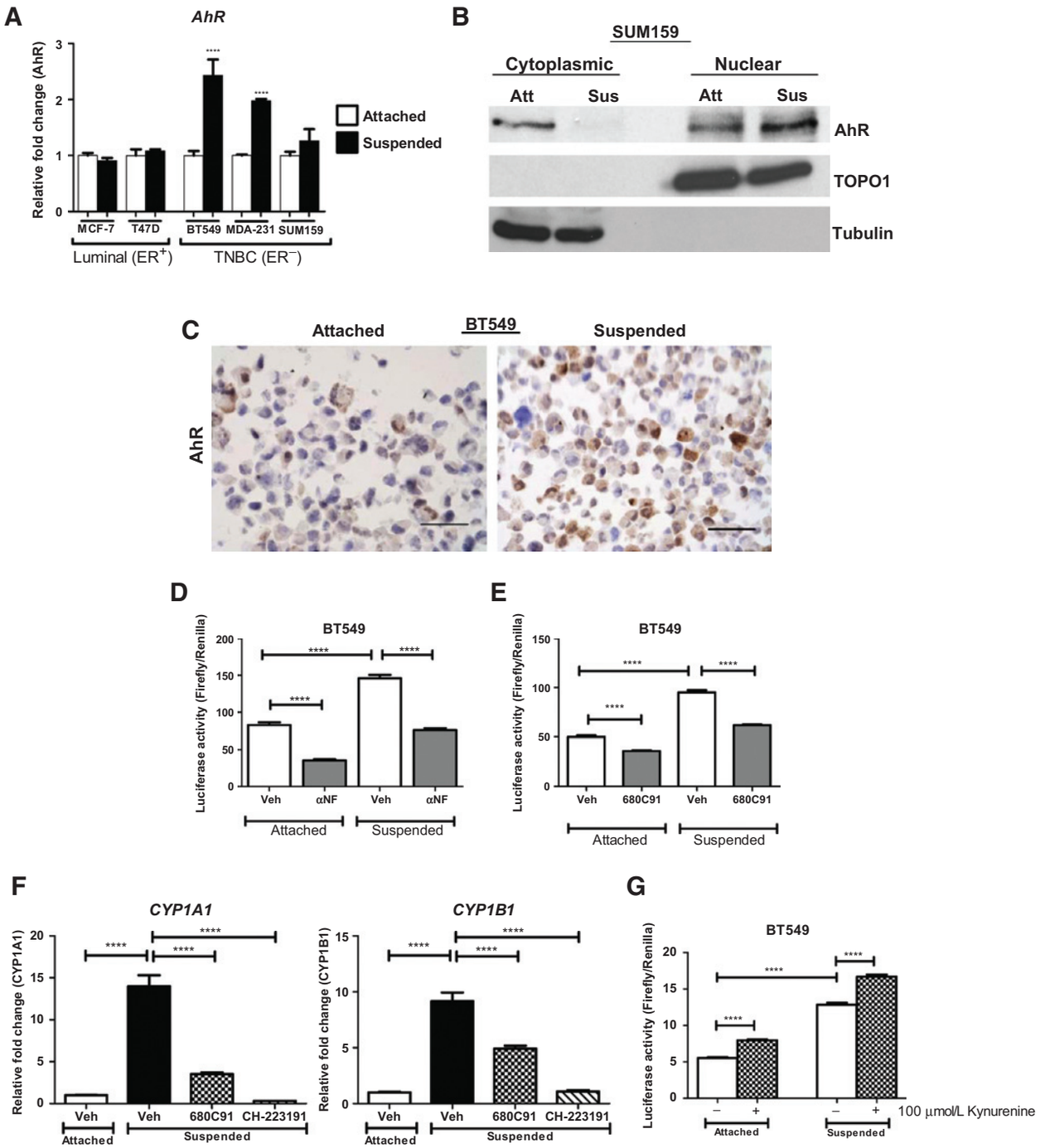

Figure 2.

AhR expression and localization are affected by suspension culture, and kynurenine activates AhR in TNBC cells in suspension. Breast cancer cell lines were plated on uncoated (attached) or poly-HEMA–coated (suspended) tissue culture plates for 24 hours. A, relative *AhR* levels measured by qRT-PCR in ER^+ or TNBC cells in attached or suspended culture for 24 hours. B, Western blot for AhR levels in nuclear and cytoplasmic fractions of SUM-159 cells in attached or suspended culture for 24 hours. C, AhR protein as detected by IHC in BT549 cell pellets grown in attached or suspended culture for 48 hours. Bar, 50 μm. D and E, AhR reporter activity in BT549 cells in attached or suspended culture for 24 hours treated with vehicle [0.1% MeOH:EA (3:1); D] or 10 mmol/L α-Naphthoflavone (AhR antagonist) or vehicle (0.1% DMSO; E) or 10 μmol/L 680C91 (TDOi). F, relative expression of AhR target genes by qRT-PCR in BT549 cells in attached or suspended culture treated with vehicle, 680C91, or CH-223191 (AhR antagonist) for 24 hours. G, AhR reporter activity in BT549 cells in attached or suspended culture for 24 hours treated with vehicle (2.5×10^{-4} M HCl) or crystalline kynurenine (100 μmol/L). *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$ by ANOVA with Bonferroni multiple comparison test.

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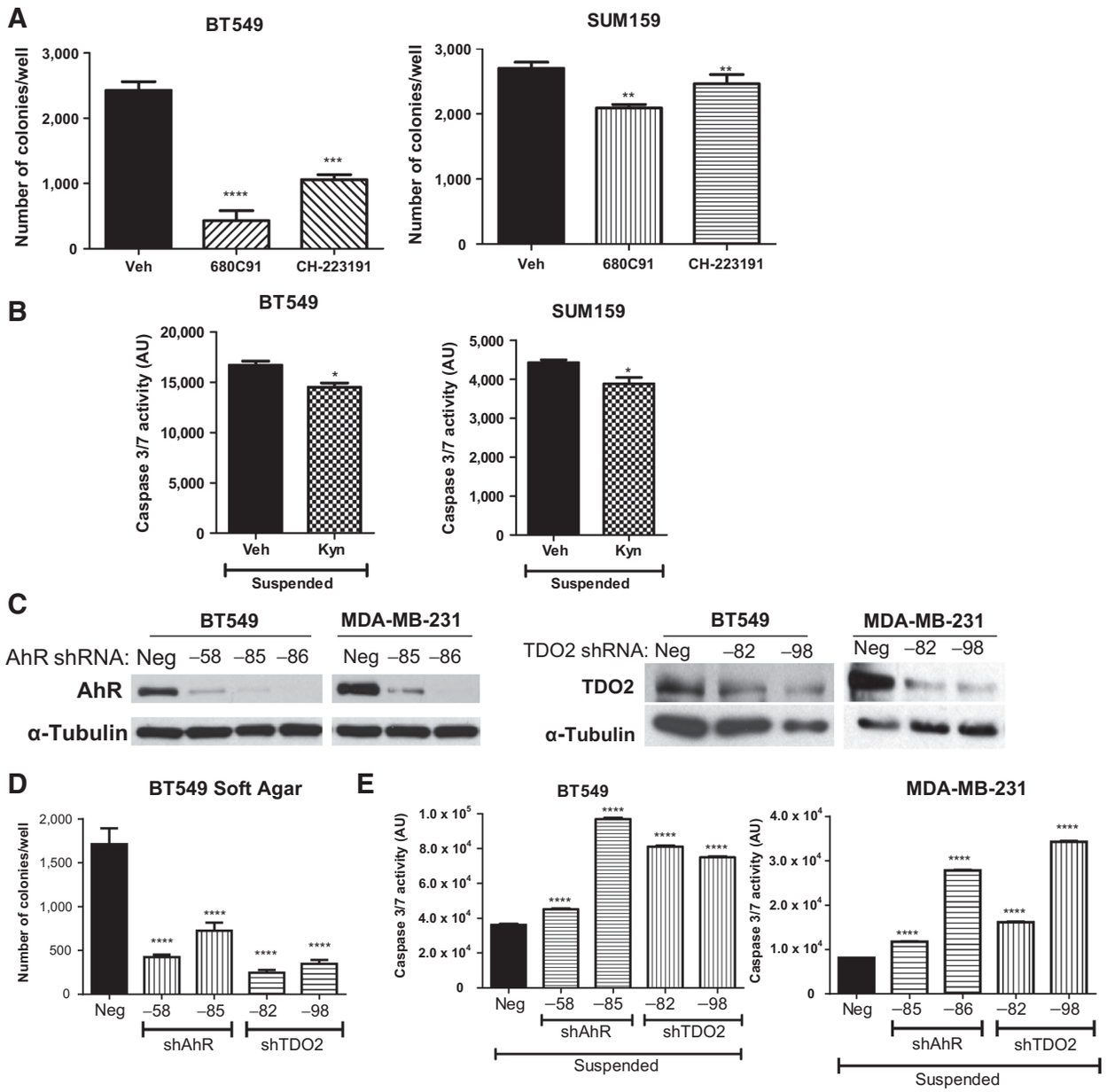

Figure 3.

Targeting the kynurenine pathway or AhR increases cell death of TNBC cells in suspension. A, anchorage-independent growth of SUM-159 or BT549 cells pretreated with vehicle (0.01% DMSO), 10 μmol/L 680C91 (TDOi), or 10 μmol/L CH-223191 (AhR antagonist) for 24 hours, then plated in 0.25% soft agar where respective treatments were maintained for 18 to 21 days. B, caspase 3/7 activity in TNBC cells (BT549 and SUM159) treated with either vehicle control or 100 μmol/L kynurenine while cultured in suspension for 48 hours. C, Western blot of TDO2 or AhR in attached BT549 or MDA-MB-231 cells following transduction with nontargeting shRNA (shNEG) or shRNA constructs targeting AhR or TDO2. D, anchorage-independent soft-agar growth of BT549 cells with knockdown of TDO2 or AhR grown in soft agar for 14 days. E, caspase 3/7 activity in BT549 or MDA-MB-231 cells transduced with nontargeting shRNA (shNEG) or shRNA constructs targeting AhR or TDO2, and plated in poly-HEMA–coated plates for 48 hours. **, $P < 0.01$; ****, $P < 0.0001$ by ANOVA with Bonferroni multiple comparison test.

To determine if TDO2 and AhR also mediate growth of TNBC cells, MDA-MB-231 and BT549 cells were treated with 680C91 or CH-223191, and cell number was measured over time. Both inhibitors significantly decreased the number of cells when grown in traditional attached conditions, and the combination was more effective than either drug alone (Fig. 4A). MDA-MB-231, BT549, and SUM159 cells were also treated with a range of 680C91 concentrations, and GI50 values were calculated as 20 μmol/L, 61 μmol/L, and 102 μmol/L, respectively (Supplementary Fig. S5). Knockdown of TDO2 or AhR similarly resulted in decreased growth of MDA-231 cells (Supplementary Fig. S6), demonstrating a role for TDO2 and AhR signaling in baseline growth of TNBC cells *in vitro* even in attached culture.

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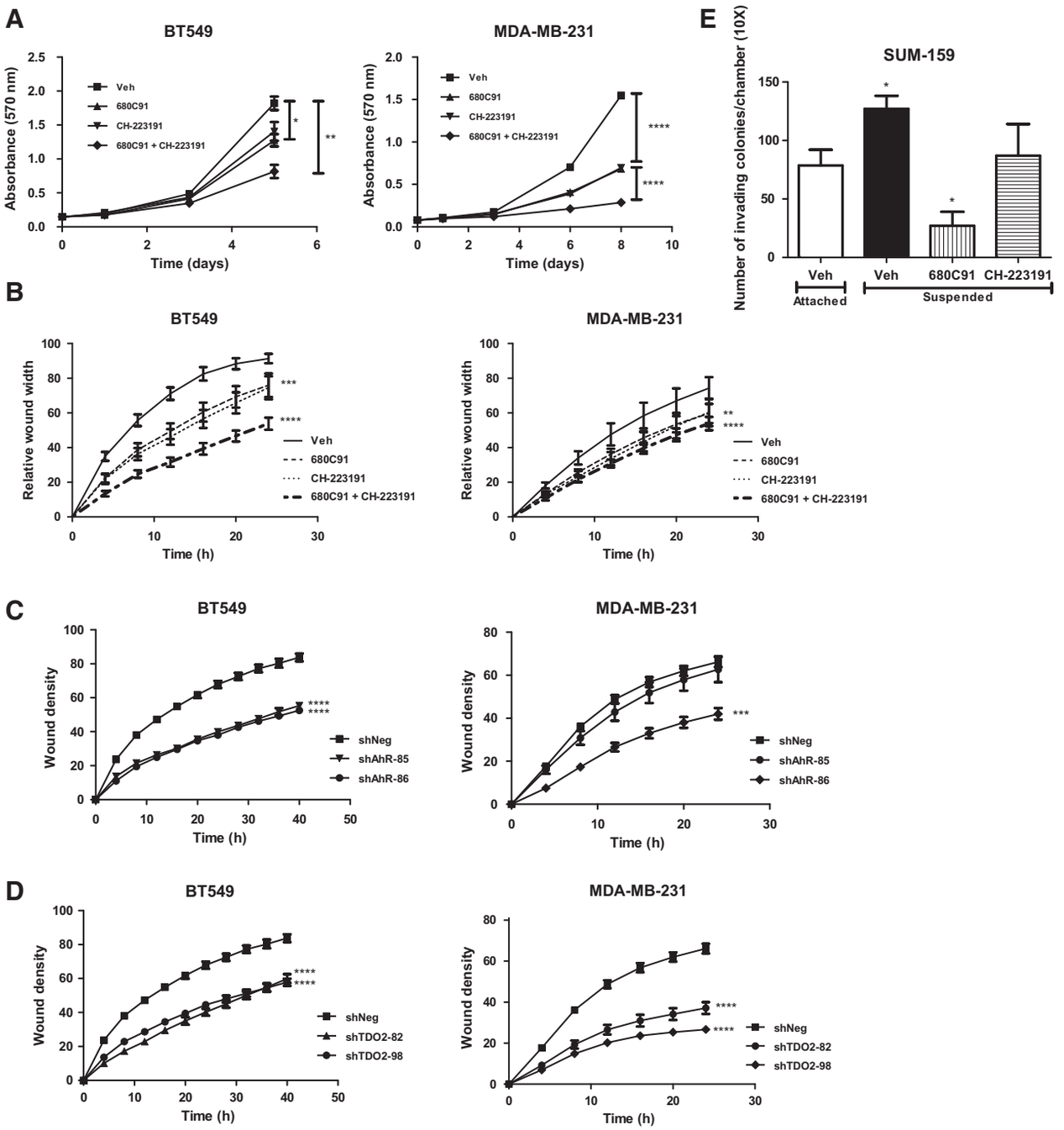

Figure 4.
TDO2 and AhR inhibition decreases cell number, migration, and invasion of TNBC cells. BT549 and MDA-MB-231 cells (TNBC) were treated with either vehicle control (0.01% DMSO), 10 μmol/L 680C91 (TDOi), 10 μmol/L CH-223191 (AhR antagonist), or in combination. A, crystal violet assay of BT549 and MDA-MB-231 cells treated for 8 days. B, scratch wound assay of BT549 and MDA-MB-231 cells treated in media containing 0.5% FBS. C and D, scratch wound assay of BT549 and MDA-MB-231 cells transduced with a nontargeting control (shNeg) or shRNAs targeting AhR (shAhR-85, shAhR-86; C) or TDO2 (shTDO2-82, shTDO2-98; D). E, Boyden chamber invasion assay of SUM-159 cells pretreated with either vehicle control (0.01% DMSO), 10 μmol/L 680C91 (TDOi), or 10 μmol/L CH-223191 (AhR antagonist) in the attached or suspended conditions for 24 hours, then seeded into the upper chamber and allowed to invade through a Matrigel-coated membrane. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$ by ANOVA with Bonferroni multiple comparison test.

In addition, pharmacologic inhibition of TDO2 and AhR each significantly reduced migration of MDA-MB-231 and BT549 cells in a scratch wound assay, and again the combination was more effective than either inhibitor alone (Fig. 4B). Knockdown of either TDO2 or AhR recapitulated this effect, significantly diminishing migration in the scratch wound assay in both BT549 and MDA-231 cells (Fig. 4C and D).

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To test whether the increased TDO2 and AhR expression observed in cells grown in suspension affects invasive capacity, SUM159PT cells were grown for 48 hours either in the attached condition, or in forced suspension culture with or without addition of 10 μmol/L of the TDO2 inhibitor 680C91 or the AhR inhibitor CH-223191. After 24 hours, 25,000 viable cells were plated in a Matrigel-coated transwell invasion chamber with continuous treatment. Cells grown in suspension for 24 hours were significantly more invasive than control cells grown in the attached condition (Fig. 4E). The addition of the TDO2 inhibitor 680C91 during suspension culture greatly decreased the invasive capacity of viable cells. Although the cells treated with the AhR inhibitor showed decreased invasion, this effect was not statistically significant (Fig. 4E).

NF-κB regulates expression of kynurenine pathway genes and *AhR* in TNBC cells

We previously demonstrated that NF-κB activity is substantially increased in TNBC cells in suspension, but not in ER^+ cells (31). Here, we again demonstrate increased NF-κB activity in TNBC cells in suspension as measured by an NF-κB luciferase reporter, and this increase is not observed using a reporter containing a mutated binding site (Fig. 5A). Interestingly, a recent global profiling study of ovarian cancer *in vivo* found that knockdown of Iκκ-epsilon, a positive regulator of NF-κB activity, decreased expression of *TDO2* and *KYNU*, suggesting NF-κB regulation of these genes (36); however, the regulatory mechanism was not directly tested.

To investigate the link between increased NF-κB activity, *TDO2*, and *KYNU*, we treated BT549 cells with a cocktail of TNFα and IL1β to activate NF-κB and found that expression of *TDO2*, *KYNU*, and *AhR* (Fig. 5B), as well as the NF-κB target genes *IL6* and *IL8* (data not shown), was significantly increased at 24 hours. Next, we tested whether inhibition of NF-κB in suspension could reduce upregulation of Kyn pathway genes and *AhR* in TNBC cells grown in suspension. Upstream inhibition of NF-κB with the Iκκ inhibitor PS1145 significantly reduced the suspension-induced increase in *TDO2*, *KYNU*, and *AhR* (Fig. 5C), as well as the NF-κB target gene *IL6* (not shown), demonstrating that NF-κB activity mediates the increased *TDO2*, *KYNU*, and *AhR* expression in TNBC cells in suspension. Lastly, a constitutively active form of IκB, a negative regulator of NF-κB, was expressed in BT549 cells, and this ablated the suspension-induced increase in *TDO2*, *KYNU*, and *AhR* (Fig. 5D). These data demonstrate that the increased transcription of *TDO2*, *KYNU*, and *AhR* in suspension is specifically induced by and dependent on NF-κB in TNBC.

TDO2 inhibition decreases metastatic colonization *in vivo*

To test the potential contribution of TDO2 activity to the metastatic capacity of TNBC cells *in vivo*, we grew luciferase-expressing MDA-MB-231 cells in forced suspension conditions for 48 hours in the presence of either vehicle control or the TDO2 inhibitor 680C91. A total of 250,000 viable cells, as determined by trypan blue staining, were then injected into the tail vein of NOD/SCID mice, and luminescence was monitored over time. Seven days after injection, mice that received vehicle-treated cells had significantly higher luminescence, and this statistically significant difference was maintained throughout the experiment (Fig. 6A and B). At the conclusion of the experiment at day 28 after injection, lung luminescence was measured *ex-vivo*, and lungs from mice that received vehicle-treated cells had significantly higher luminescence compared with mice that received cells treated with the TDO2 inhibitor (Fig. 6C). A significant decrease in the number of metastatic nodules in the lungs from mice receiving cells treated with 680C91 was also observed by hematoxylin and eosin (H&E; Fig. 6D). Together, these data demonstrate that TDO2 inhibition decreases the ability of TNBC cells to successfully metastasize following tail vein injection *in vivo*.

***TDO2* is more highly expressed in ER^- than ER^+ breast cancer and correlates with poor prognosis**

To determine the clinical relevance of our findings, we examined TDO2 expression in primary TNBC samples by IHC. As shown in Fig. 7A, primary TNBC were strongly positive for TDO2 expression. We evaluated *TDO2* gene expression in patient samples using publicly available gene expression microarray datasets from Oncomine. The Curtis and colleagues dataset (37) had the largest patient population (n = 1,998) and was therefore selected for analysis. Notably, *TDO2* was the sixth most highly overexpressed gene in breast carcinoma compared with normal breast tissue (top 1% of overexpressed genes, $P < 0.0001$; Fig. 7B). *TDO2* gene expression was significantly higher in ER-negative breast tumors compared with ER-positive tumors ($P < 0.0001$; Fig. 7C). High *TDO2* expression was also associated with increasing grade ($P < 0.001$; Fig. 7D), supporting the hypothesis that tumors with high *TDO2* expression may have increased metastatic potential. Similarly, *IDO1* was overexpressed in breast carcinoma compared with normal breast tissue, although it was not as highly overexpressed as *TDO2* (Supplementary Fig. S7A). *IDO1* was also more highly expressed in ER^- than ER^+ breast tumors in both the Curtis and TCGA datasets (Supplementary Fig. S7B and S7C).

Finally, patients whose tumors had above-median *TDO2* expression had approximately 3 years shorter overall survival when compared with those with below-median *TDO2* expression (median overall survival 10.62 years vs. 13.31 years, respectively; $P = 0.0002$, log-rank test; Fig. 7E), suggesting that *TDO2* expression may contribute to tumor progression and poor prognosis. Similarly, patients with above-median *IDO1* expression had a shorter survival than those with lower *IDO1*, but the difference in survival was not as large (Supplementary Fig. S7D). This further supports the importance of targeting TDO2 in breast cancer patients, possibly in combination with IDO1, rather than targeting IDO1 alone as in current clinical trials.

Discussion

TNBC has no effective targeted therapies and the worst prognosis of the breast cancer subtypes, due in part to its propensity for rapid metastasis (3). Indeed, patients with metastatic TNBC have a median survival of only 13 months (5). However, few studies have examined pathways that support anoikis resistance in TNBC. In this study, global gene expression profiling revealed the novel finding that TNBC cells in forced suspension upregulate two related pathways: AhR signaling and the kynurenine pathway of tryptophan catabolism. Indeed, global metabolomic profiling identified formylkynurenine and Kyn as the most highly upregulated intracellular metabolites, and Kyn as the third most-highly

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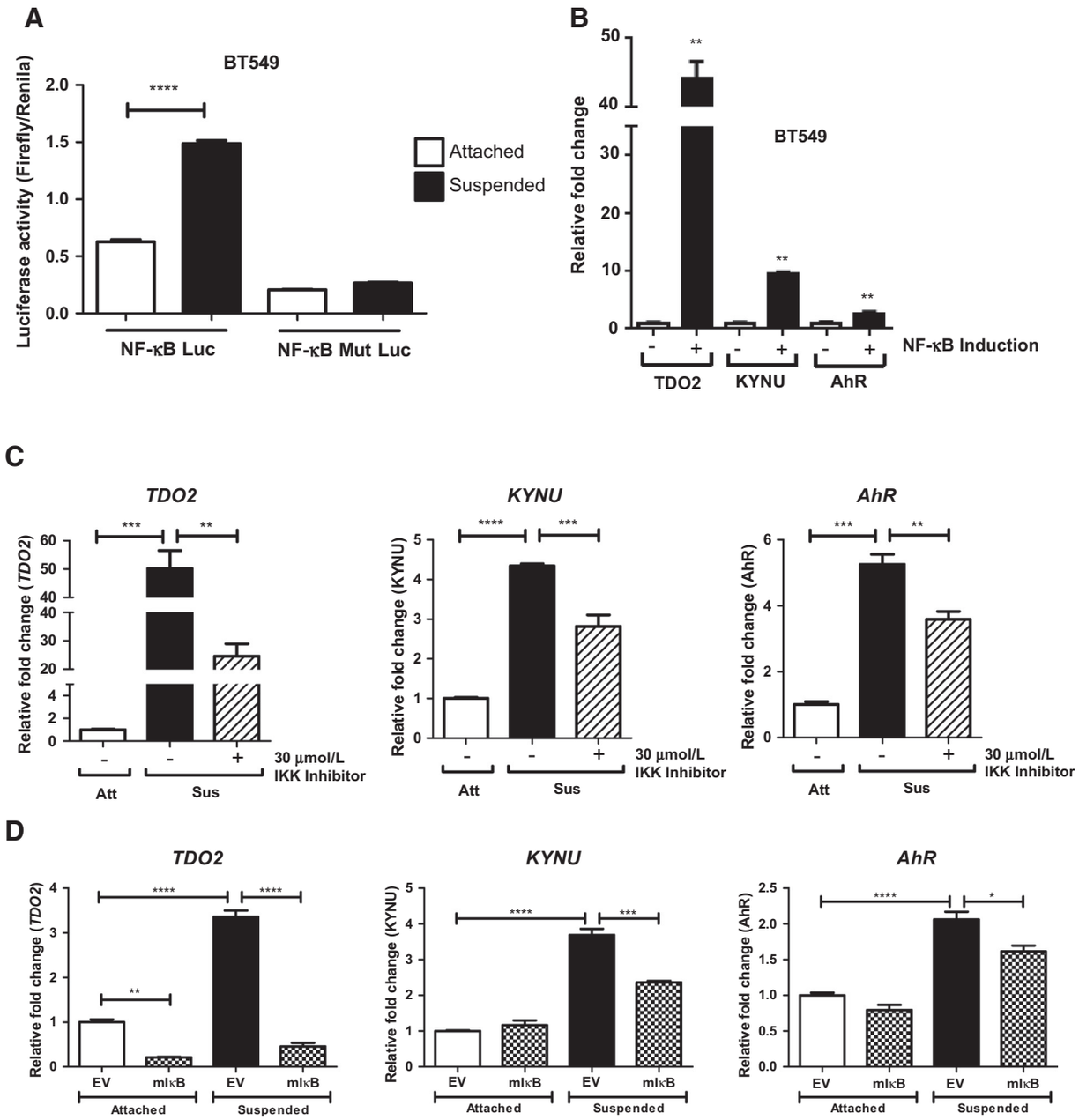

Figure 5.

NF-κB regulates expression of TDO2, KYNU, and AhR in TNBC cells in suspension. A, activity of an NF-κB (left) or mutated NF-κB (right) luciferase reporter in BT549 cells in attached or suspended condition for 24 hours. B, relative expression of *TDO2, KYNU*, and *AhR* by qRT-PCR in BT549 cells treated with vehicle or TNFα (10 ng/mL) plus IL1β (10 ng/mL) for 24 hours. C, relative expression of *TDO2, KYNU*, and *AhR* by qRT-PCR in BT549 cells grown in attached or suspended culture treated with vehicle (0.01% DMSO) or 30 μmol/L PS1145 (IKK inhibitor) for 24 hours. D, relative expression of *TDO2, KYNU*, and *AhR* as measured by qRT-PCR in BT549 cells grown in attached or suspended culture and expressing a stable empty-vector (EV) or mutant (constitutively active) IκB expression vector. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ by ANOVA with Bonferroni multiple comparison test.

increased secreted metabolite in conditioned media from cells in forced suspension.

Based on our data demonstrating that TNBC cells in suspension upregulate AhR and TDO2, increase secretion of Kyn, and rely on TDO2 and AhR for anchorage-independent growth and invasive potential, we hypothesized that suspended TNBC cells utilize an autocrine signaling loop in which Kyn activates AhR to support anoikis resistance and anchorage-independent growth, as well as migration and invasion (Fig. 7F). Central to this model is upregulation of TDO2, a key enzyme in the Kyn pathway. Our observation that pharmacologic inhibition of TDO2 decreased the ability of MDA-MB-231 cells to form lung

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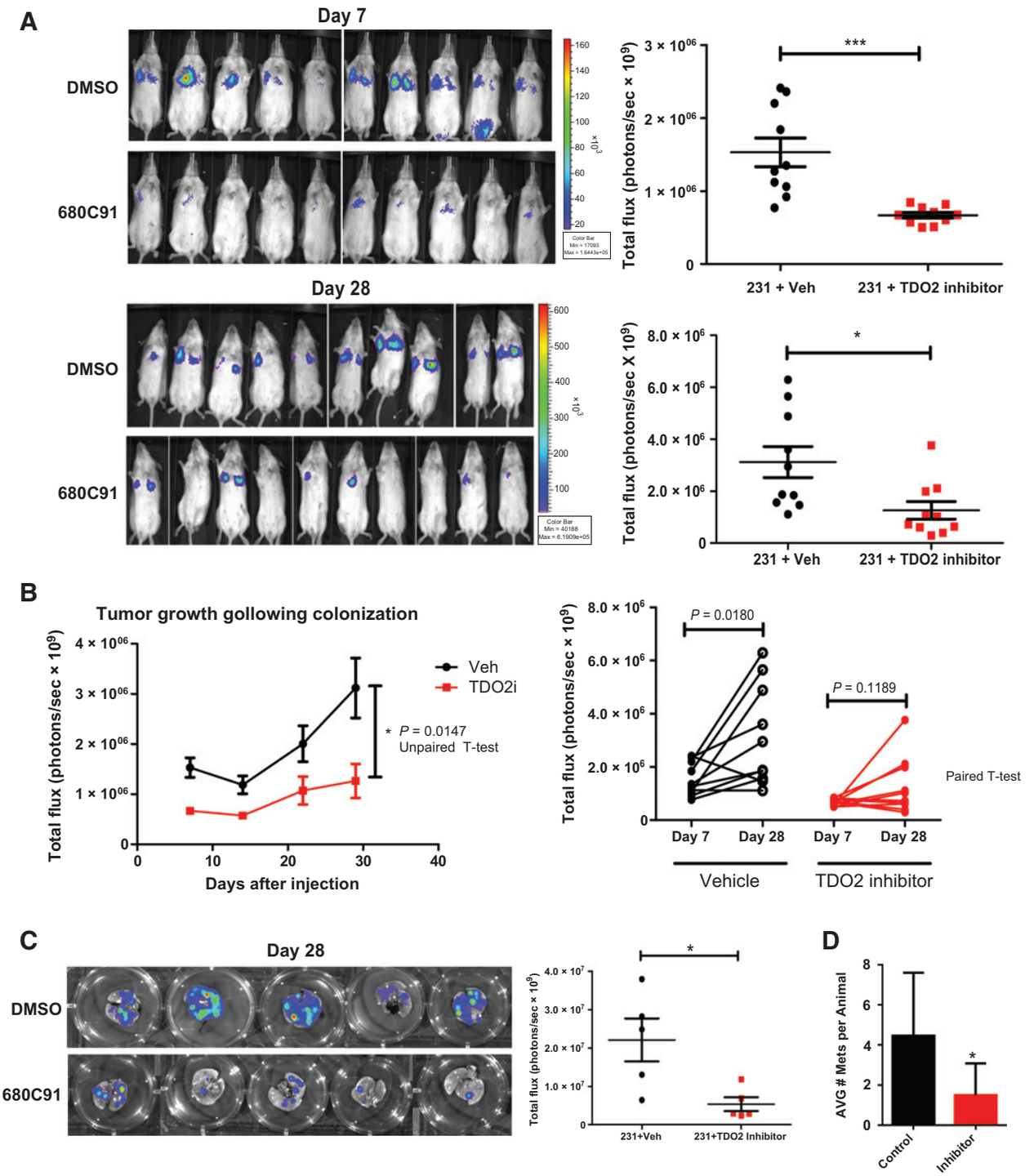

Figure 6.
Targeting TDO2 decreases colonization and outgrowth of TNBC cells *in vivo*. Luciferase-expressing MDA-MB-231 cells were pretreated for 48 hours in forced-suspension culture with either vehicle (0.01% DMSO) or 10 μmol/L 680C91 prior to tail vein injection. A and B, metastatic colonization was measured by whole animal IVIS imaging at 7 days after injection and weekly thereafter. C, *ex vivo* imaging of lungs 28 days after injection. D, quantification of lung metastases based on H&E of FFPE lung sections. *, $P < 0.05$; ***, $P < 0.001$ by two-tailed *t* test.

metastases *in vivo* suggests that TDO2 inhibition represents a novel opportunity for targeted therapy to inhibit TNBC metastasis.

Further, we show that the Kyn pathway components TDO2 and KYNU, as well as AhR, are regulated by the transcription factor NF-κB in suspension. We previously identified a different

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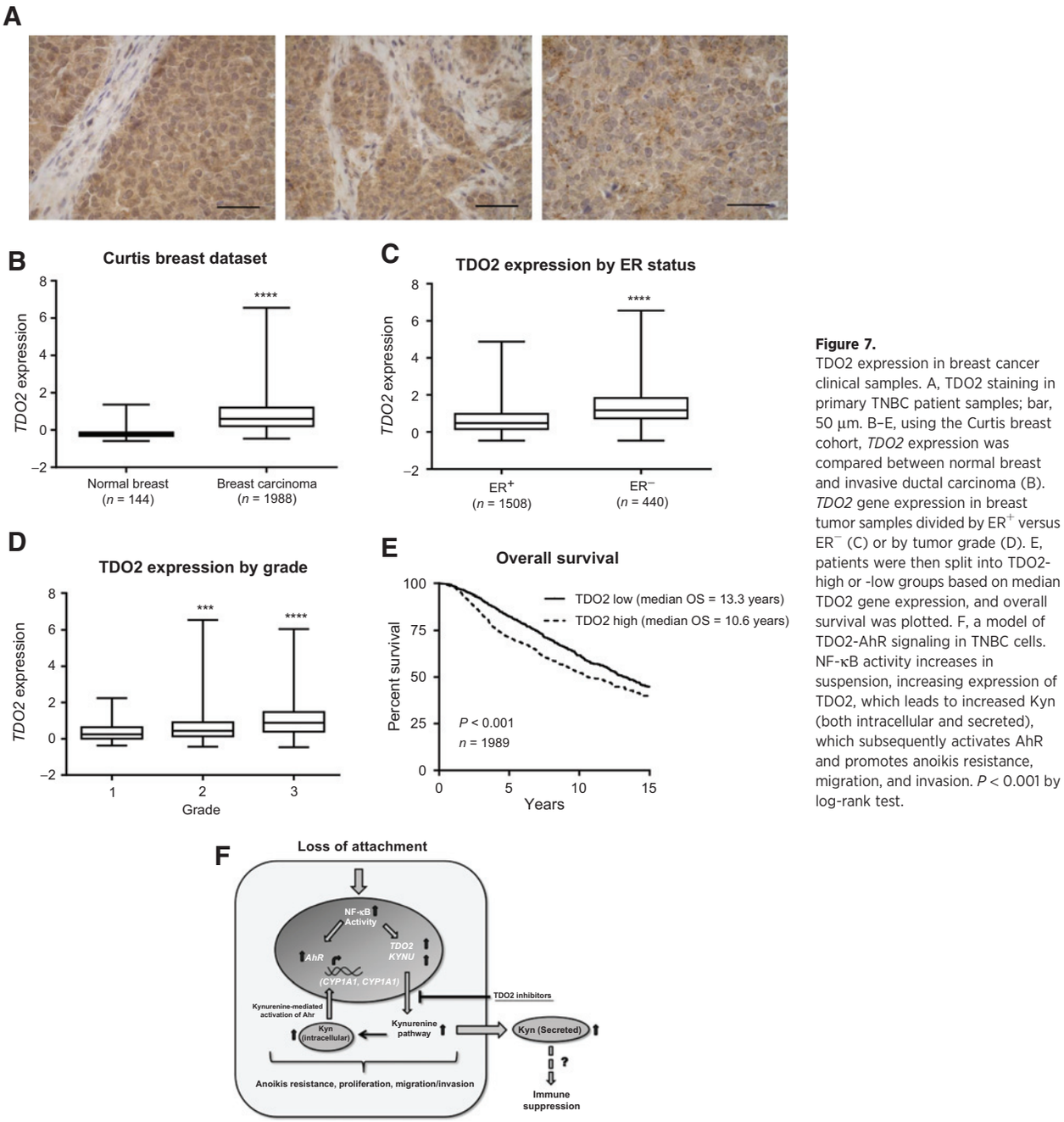

Figure 7.
TDO2 expression in breast cancer clinical samples. A, TDO2 staining in primary TNBC patient samples; bar, 50 μm. B–E, using the Curtis breast cohort, *TDO2* expression was compared between normal breast and invasive ductal carcinoma (B). *TDO2* gene expression in breast tumor samples divided by ER^+ versus ER^- (C) or by tumor grade (D). E, patients were then split into TDO2-high or -low groups based on median TDO2 gene expression, and overall survival was plotted. F, a model of TDO2-AhR signaling in TNBC cells. NF-κB activity increases in suspension, increasing expression of TDO2, which leads to increased Kyn (both intracellular and secreted), which subsequently activates AhR and promotes anoikis resistance, migration, and invasion. $P < 0.001$ by log-rank test.

NF-κB–regulated autocrine signaling loop involving the neurotrophic tyrosine kinase, type 2 (NTRK2 or TrkB), and the TrkB ligand neurotrophin 3 (NTF3) that promotes survival of TNBC cells in suspension (31), suggesting that increased NF-κB signaling in suspended cells supports multiple prometastatic attributes of TNBC.

In addition to its autocrine role affecting growth, migration, and invasion of tumor cells that we describe here, the Kyn pathway may also facilitate metastasis through paracrine signaling mechanisms involving suppression of immune surveillance. Specifically, TDO2-mediated Kyn production prevented T-cell–mediated immune rejection of the immunogenic P815 mouse tumor cell line in immunized mice (38). In dendritic cells, IDO1 activity can lead to the AhR-dependent generation of regulatory T cells via a coordinated increase in Kyn and decrease in tryptophan (33). Inhibition of IDO1 also sensitizes cancer cells to chemotherapy in a T-cell–dependent manner (39).

Multiple clinical trials testing the efficacy of Indoximod (1-methyl-D-tryptophan), an IDO1/2 inhibitor, are currently under way, including one in metastatic breast cancer in combination with conventional chemotherapies or immunotherapy (NCT01792050). These trials are based on the ability of IDO1/2 inhibition to reverse immune suppression and enhance the antitumor immune response. However, 1-MT does not target TDO2 (40). Importantly, our data combined with data mined from publicly available breast cancer clinical cohorts suggest that TDO2 may be the more relevant target in TNBC. Interestingly, the membrane protein responsible for tryptophan import into cells, LAT1, is also more highly expressed in TNBC than in other breast cancer subtypes (41). Although a recently published analysis of serum metabolite concentrations in breast cancer patients found no difference in tryptophan levels between ER^+ and ER^- breast cancer patients, median levels of Kyn were significantly higher in patients with ER^- tumors (42). Although this finding was attributed to IDO1 activity, we find that that *TDO2* is higher in ER^- tumors than ER^+ in two publicly available datasets, and that *TDO2* expression is higher than *IDO1* at baseline in two of three TNBC cell lines tested and *TDO2* increases more strongly in suspension in all cell lines. Lastly, and perhaps the strongest indicator of the relative role of TDO2 versus IDO1, blocking TDO2 activity completely prevented the detachment-induced increase in Kyn secretion observed in TNBC cells in suspension (Fig. 1F).

Collectively, our results suggest that in TNBC, TDO2 is an important contributor to Kyn production, and thus should be considered as a therapeutic target. High *TDO2* in primary breast tumors from publicly available datasets correlates with shorter overall survival. *TDO2* was significantly upregulated in metastases of leiomyosarcoma patients compared with primary tumors (43), and increased *KYNU* expression is associated with metastasis of breast cancer cells to the lung and brain (44, 45). In addition to abrogating the autocrine protumorigenic effects of Kyn, TDO2 inhibition may also inhibit Kyn activation of AhR in immune cells and thereby reduce TNBC immune evasion, but this remains to be tested.

Although we demonstrate a significant increase in Kyn levels in TNBC cells in suspension, it is possible that the effects of TDO2 could be mediated in part through production of other intermediate molecules or NAD^+ production. However, AhR nuclear localization and transcriptional activity are clearly increased in TNBC in suspension culture, and other recent studies find that AhR affects growth and migration of breast cancer cells (27, 28) and castrate-resistant prostate cancer cells (46). Studies examining AhR expression in breast cancer primary tumors found that higher AhR expression correlates with good prognosis (47). However, our data suggest that AhR expression, nuclear localization, and transcriptional activity may be low until cells begin the metastatic process by detaching from the primary tumor. Thus, AhR in the primary tumor may not reflect its role in supporting the metastatic cascade.

The independent tumor-promoting roles of the Kyn pathway (particularly via IDO1) and AhR signaling have been recognized separately for many years. Recently, the connection of these two pathways via Kyn serving as an AhR ligand was discovered (12, 33–35). Our study demonstrates that the TDO2-AhR signaling axis may be of particular importance in TNBC because it is strongly activated in this highly metastatic breast cancer subtype in response to detachment, and supports anchorage-independent survival and invasive capacity. The combination of protumorigenic autocrine effects and potential immunosuppressive paracrine signaling effects makes this pathway an attractive target for therapeutic intervention. Our findings that inhibition of the Kyn or AhR pathways decreased *in vitro* characteristics associated with multiple steps in the metastatic cascade, and that pharmacologic inhibition of TDO2 decreased TNBC metastasis *in vivo*, suggest that inhibitors of TDO2 may represent an exciting opportunity for targeted therapy for TNBC to decrease mortality from this aggressive breast cancer subtype.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N.C. D'Amato, L.I. Greene, D.R. Cochrane, N.S. Spoelstra, T.G. Nemkov, A. D'Alessandro, J.K. Richer

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N.C. D'Amato, T.J. Rogers, M.A. Gordon, L.I. Greene, N.S. Spoelstra, T.G. Nemkov, K.C. Hansen, J.K. Richer

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A TDO2-AhR Signaling Axis Facilitates Anoikis Resistance and Metastasis in Triple-Negative Breast Cancer

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